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EDITORIAL NOTE.

The papers included in this volume are published for the first time, and represent, with the exception of Professor Ehrlich's contribution, work carried out in the laboratories of the Institute.

The papers are published with the authorisation of the Council of the Institute, and I am indebted to Sir Henry Roscoe and Mr. S. G. Shattock for valuable assistance and advice.

ALLAN MACFADYEN,
Director.

August, 1899.

CONTENTS.

PAGE

Observations upon the Constitution of the Diphtheria Toxin. By Professor P. Erhlich 1

On a new Pathogenic Streptothrix. By George Dean, M.B. 17

A Contribution to the Study of Diphtheria Toxin. By William Bulloch, M.D. 46

The Bacteriology of the Normal Conjunctival Sac, from a Report of 200 Cases, and its practical bearing on the utility of antiseptics in Ophthalmic Surgery. By Arnold Lawson, F.R.C.S.Eng. 56

Preliminary Observations on the Occurrence of the Bacillus Enteritidis Sporogenes (Klein) in Ulcerative Colitis and in the Normal Dejecta. By Richard T. Hewlett, M.D. 70

Photogenic Bacteria. By E. T. Barnard 81

The Pathogenicity of the Pseudo-Diphtheria Bacillus and its Relation to the Klebs-Loeffler Organism. By Alfred Salter, M.D. (Lond.) ... 113

The Fermentation of Sugars by Bacillus Coli Communis and Allied Organisms. Part I. By Arthur Harden, M.Sc. 126

Observations upon the Structure of Bacteria. By Sydney Rowland, M.R.C.S. 143

Thermophilic Bacteria. By Allan Macfadyen, M.D., and Frank R. Blaxall, M.D. (second paper) 162

The Technical Applications of Bacteriology. By G. Harris Morris, Ph.D., F.I.C.... 188

The Pathology of Cancer: An Experiment. By H. Lambert Lack, M.D. 198

On Neisser's Diagnostic Stain for the Diphtheria Bacillus. By R. T. Hewlett, M.D. 201

An Instance of Symbiotic Fermentation. By Allan Macfadyen, M.D. 207

On some Organisms of the Bacillus Coli Communis group isolated from Drinking-water, &c. By Joseph Lunt, B.Sc, ... 219

Action of Hydrogen Peroxide and the Oxides of Copper on Formaldehyde. By Arthur Harden, M.Sc. 232

Laboratory Notes:—

I.—Examinations of Anthrax 238

II.—A Case of Natural Prodigiosus Infection 239

III.—Tropical Abscess of the Liver 239

IV.—The Early Diagnosis of Diphtheria 240

V.—Table Showing the Age-Incidence of Diphtheria 241

VI.—Flagella Staining 242

VII.—Capsule Staining 244

VIII.—Disinfection on a large scale with Formalin 244

Notes on Apparatus:—

I.—A New Hot Stage for the Microscope 246

II.—A New Photomicrographic Apparatus 248

III.—A Method of rapidly Disintegrating Micro-organisms 250

IV.—A New Microscope Lamp 252

LIST OF ILLUSTRATIONS.

(The illustrations are mainly from photographs by Mr. J. E. Barnard.)

		PAGE
The Jenner Institute of Preventive Medicine	Plate facing	ix.
The Bacteriological Laboratory	„	xi.
The Chemical Laboratory	„	xii.
A Research Room	„	xiv.
On a New Pathogenic Streptothrix	Plates facing	45
Photogenic Bacteria	„	112
The Pathogenicity of the Pseudo-Diphtheria Bacillus and its Relation to the Klebs-Loeffler Organism	Plates facing	125
Observations upon the Structure of Bacteria ..	Plate facing	161
Thermophilic Bacteria	Plates facing	187
A New Hot Stage for the Microscope	.. .. ..	247
A New Photomicrographic Apparatus	Plate facing	249
A Method of rapidly Disintegrating Micro-organisms	.. .. ..	251
A New Microscope Lamp	.. .. ..	252

THE JENNER INSTITUTE OF PREVENTIVE MEDICINE.

INTRODUCTION.

THE opportunity was taken in connection with the publication of the first volume of the Transactions of the Institute to give a full account of the objects for which the Institute was established, and to state the progress that had been made in their fulfilment since its incorporation in 1891.

Whilst the work of the Institute has continued to make satisfactory progress, the events of the past year have been amongst the most notable in the history of its affairs.

An event of far-reaching importance as regards the future development of the Institute was the public announcement of Lord Iveagh's intention to devote the sum of £250,000 to the promotion of the objects for which the Institute was founded. Certain conditions were attached by Lord Iveagh to the above gift. These were carefully considered by the Council and accepted by the members of the Institute. Under the new Articles of Association, which await the consent of the Court, the affairs of the Institute will in future be controlled by a governing body consisting of seven members, one of such members to be appointed by the Royal Society, three of such members by the Council of the Institute, and the remaining three members by Lord Iveagh or his representatives.

The Council, though ceasing to be the directly administrative body, will still continue to exercise a valuable influence over the affairs of the Institute through the three members it will annually elect to the governing body and the advice it may be called upon to give from time to time. Lord Lister has consented to act as the chairman of the new governing body when constituted.

Through Lord Iveagh's munificence the objects of the Institute can now be developed on a scale commensurate

with their importance, and the aim of its founders to establish a national home for bacteriological work and inquiry adequately realised.

The Jenner Memorial Committee having decided to transfer the funds in its possession to the British Institute of Preventive Medicine, steps were taken to ensure an adequate fulfilment of the laudable object of the fund, viz., to perpetuate the memory of Jenner and his work. With this intention the members of the Institute at a special general meeting unanimously resolved that the Institute be in future termed the "Jenner Institute of Preventive Medicine." This change in title was duly effected under a licence from the Board of Trade, dated 6th December, 1898.

The first series of Transactions mainly embodied researches carried out at the temporary premises of the Institute in Gt. Russell Street, where, on a small scale, the various departments had been successfully initiated. In May, 1898, the Institute took possession of the new building at Chelsea, and the laboratories are now fully equipped and at work. It may therefore be of interest to give a short account of the arrangements that have been made for the work it is proposed to carry on. The accompanying illustrations are from photographs of the building and the main laboratories.

The new building has been erected on a site on the Thames Embankment, near Chelsea Bridge, from designs by Messrs. A. Waterhouse & Son. The building is completely detached, and stands on its own grounds, which were acquired from the Duke of Westminster, who is a member of the Council of the Institute.

Administration.

The board room and general offices, together with a waiting room, are situated on the ground floor, near the main entrance, to the right of which is the Director's private office, together with two laboratories for himself and his assistants. These laboratories will accommodate six workers and are fitted up with the usual appliances for bacteriological investigation. A lift is provided for goods and

THE JENNER INSTITUTE OF PREVENTIVE MEDICINE.
THE BACTERIOLOGICAL LABORATORY.

passengers, and there is lavatory accommodation on every floor.

The Institute has been placed on the Central Telephone Exchange and the Boy Messenger Service, whilst the various departments are connected by means of an inter-communicating telephone.

Bacteriological Laboratories.

The main bacteriological laboratory is situated at the back, on the ground floor, and occupies the entire length of the building. It has accommodation for twenty-five students, and has the advantage of a north light for microscopical work. The working benches are of teak, impregnated with paraffin and are provided with gas, water and electric light. Each worker is provided with a complete set of apparatus for his work, as well as with a set of drawers and lock-up cupboards. For dissections and aseptic operations a slate-topped table is provided. The incubators stand on slate slabs, and a special glass blowing table has been provided. At one end of the room is a fume chamber, and a wash-up sink with hot and cold water. A small room in connection with this laboratory will admit of operations being carried out under the best conditions for aseptic work. The floor is concrete, the benches are of slate, and the walls covered with opalite. In this laboratory courses of instruction are given. The Institute has already attracted workers from all parts of the world, and the number will no doubt greatly increase with the enhanced facilities of its new building. The courses of instruction are attended by qualified medical men, army surgeons and students of hygiene. A course of systematic bacteriology is given comprising lectures and practical work. The practical instruction extends over three months, and touches on all the phases of bacteriological work. This laboratory is also utilised to give those desiring it a thorough training in bacteriological methods, with a view to subsequent research work.

A room in the basement has been specially fitted up for the preparation of the culture media. There is a stone

bench with hood for the steam sterilisers, stone benches for the autoclaves and serum inspissators, tables, shelves, &c., two large sinks and a large copper with counterpoised top for boiling out used tubes and plates. In this room the student will be instructed in the methods of the preparation of the various culture media, such preparation of media being regarded as an essential part of the training. There is also a special room which will be maintained at incubation temperature. The convenience of this will be recognised by all bacteriological workers. Such a room will be particularly useful for the preparation of tuberculin, mallein and similar fluids on a large scale. There is, further, a large room in the basement which will be fitted up as a workshop for general repairs. The same room contains a large centrifugal machine with electric motor, and a water distilling apparatus. An adjacent room contains a cold storage installation. The machine is charged with ammonia as the refrigerating agent. The room contains a brine tank, in which is a vertical coil and the tank is kept at about 5° F. below freezing point. This is sufficient to keep the entire room at a temperature of 30 to 40° F. with ease. There is, in addition, a cupboard containing a small brine tank, with a flat coil at the bottom, on which and in the brine, flasks, bottles, &c., can be stood, and kept at a temperature from freezing point to 10° below. There is convenient cellar accommodation for the storage of chemicals and apparatus, and provision is made for disinfecting the blouses, &c., of the workers, whilst all necessary laundry work will be done on the premises.

The Photographic Laboratory.

The back of the basement is devoted to photography. In a large room are provided two complete photomicrographic cameras, one for use with the electric arc light, and constructed to carry out work with the highest powers, and the other to be used for lower power work, and also for purposes of preliminary instruction. The larger form of apparatus is of the latest design, with some new features introduced to facilitate its working. Thus, to ensure firmness and freedom from vibration there are two brickwork

THE JENNER INSTITUTE OF PREVENTIVE MEDICINE.
THE CHEMICAL LABORATORY.

and masonry supports for the cameras, each of which is so placed that a curtain can be drawn at any moment to effectually screen off either apparatus. Attention will be given to the application of photography to spectroscopy, so far as it is applicable to bacteriological research, and apparatus will also be provided for photometric observations. The dark room contains two large sinks, with sprays for washing purposes. A cabinet of photographic slides is in process of formation, and the necessary illustrations to scientific papers will be prepared in this laboratory.

The Chemical Laboratory.

The large chemical and water laboratory is situated on the first floor. It contains all appliances for chemical work and working accommodation for twenty students. The two fume chambers contain water and gas supply, which can be regulated without opening the chambers. In these distilling and evaporating operations can readily be carried on. There is also a special hooded chamber for water baths. The teak benches have ample water and gas supply, as well as taps and wastes, for distilling purposes, and an electric light supply. The central bench also contains an experimental main, which will allow of electric currents of any strength being used. This main is regulated by means of a motor transformer in the basement. Provision is likewise made in the basement for elementary organic analyses in the shape of a combustion furnace. In this room investigations of a chemical character and bearing on hygiene and preventive medicine will be conducted, and it is hoped the laboratory will become a centre for the investigation of matters concerning public health. It is intended that the general training given in chemistry and physics shall be of a thorough character and compare with that given in hygienic institutes on the Continent. A small meteorological station is connected with the laboratory. The apparatus for gas analyses and the polariscope are placed in a separate room. At the back of the building, on the same floor, are rooms for private research work and the balance room.

The Hansen Laboratory.

A notable addition has been made to the departments of the Institute by the foundation of the Hansen Laboratory for the study of the practical application of bacteriology to industrial and technical processes. A knowledge of the biology of fermentation is of great importance to the brewer, the chemist, and agriculturist, and little or no encouragement has hitherto been given to this branch of investigation in this country, from which the most important results may be anticipated in the future. The laboratory is situated on the first floor, and is completely equipped for research and instruction in the biology and chemistry of fermentation. The department was opened for work early in December last, and there is every reason to believe that students who now go to the Continent for instruction will avail themselves of the advantages it offers. The laboratory is provided with a working model of the Hansen apparatus used for the cultivation of pure yeast on an industrial scale.

Private Research.

A series of rooms have been fitted up on the second floor with every convenience for private research work. There is likewise an experimental room, and facilities for investigations of a bacteriological chemical nature.

On the same floor are the laboratories and office at present used by the Local Government Board for the preparation and storage of glycerinated calf-lymph.

Museum and Lecture Theatre.

The museum is situated on the third floor. It consists of a large room with a gallery and two bays. A commencement has been made with a series of museum specimens intended to illustrate the work of the Institute. Attached to the museum is a preparation room. The lecture theatre is situated at the back of this floor and has accommodation for 150. The room can be darkened, and the lecture-table is designed to afford facility for demonstrations. It is furnished with a sink, water, electric light, and a terminal of the experimental main. Ample provision is also made

THE JENNER INSTITUTE OF PREVENTIVE MEDICINE.
A RESEARCH ROOM.

The whole block of buildings is lighted electrically, the current being taken from the mains of the local company. *Current* is supplied for experimental purposes by a distinct system of mains, which are carried into the various laboratories to terminals to which temporary connection can readily be made. This main is fed by a motor transformer, taking current from the supply at a pressure of 200 volts, and allowing it to be supplied at any pressure from 5 to 50 volts. The entire current can be cut off from each floor of the building by means of switches placed in the corridors.

The warming and ventilation of the building are provided for by means of a moist air heating apparatus which is placed in the basement.

A commencement has been made with the foundations of the new wing which, when erected, will complete the original scheme of the building.

The department for the preparation of antitoxine is situated at Sudbury, near Harrow.

The accompanying illustrations show the front elevation of the Institute, the main Pasteurian and the main Laboratories, and a Research Room

TRANSACTIONS

OF THE

JENNER INSTITUTE OF PREVENTIVE MEDICINE.

OBSERVATIONS UPON THE CONSTITUTION OF THE DIPHTHERIA TOXIN.[1]

BY PROFESSOR P. EHRLICH.

(*From the Institute for Serumprüfung und Serumforschung, Steglitz, Berlin.*)

IMMUNITY is a reaction of the living organism towards a given class of poisons, in which are included the toxins, toxalbumins and ferments. The study of the antitoxin producing poisons consequently affords us the best possibility for elucidating this complicated and important phenomenon. There is not much to be expected at present from purely chemical methods of investigation, at least the history of the ferments, which are most closely related to the toxins, does not encourage one to place any implicit reliance upon research in this direction. Our primary duty, therefore, as medical investigators, is to search for a solution of such questions in the field that properly belongs to us—that of biological experiment. Progress was not to be hoped for so long as no clear views prevailed with regard to the essential nature of the antitoxin action and the opinions of the most

[1] Professor Ehrlich has been good enough to write the above paper in compliance with my suggestion that a general survey from his own pen of the results he has reached and which are recorded in many individual papers would be most helpful to his English-speaking colleagues. It is with his full concurrence that I undertook an English translation.—A. MACFADYEN.

competent inquirers differed widely, and so long as the problem was not decided whether the antitoxin acted chemically and directly on the toxin or indirectly in virtue of a species of cell immunisation. The first step which I was led to take in this direction consisted in excluding the animal experiments—so difficult to interpret—and in creating simple experimental methods, which would allow, if I might so express it, of work under purely chemical conditions. In this way the experiments *in vitro* originated, by which I was able to furnish the proof that in the case of ricin and abrin, poison and anti-poison act directly on each other. The significance of these investigations, which were speedily confirmed and expanded by Kossel, Camus and Gley, and Kanthack, is apparent, inasmuch as a direct chemical interreaction of the two components was proved and the vital theory, as upheld by Roux and Buchner, became untenable. At the same time the essential nature of the process, whether it consists in a destruction of the poison or a mutual combination of the two components, is not at all evident from these experiments. And the recent and valuable experiments of Martin and Cherry, in which mixtures of snake-venom and antivenene were filtered under pressure through gelatine membranes, only prove that snake-venom disappears in the mixture without giving any clue as to the action that takes place. I cannot, therefore, agree with Behring when he considers that these experiments demonstrate a chemical combination to have occurred.

At the time when the doctrine of immunity was in its initial stages it was assumed that the antitoxin destroyed the toxin, somewhat after the manner of a ferment. This point of view has, however, been gradually abandoned, and the suggestion that both components unite chemically now finds many supporters. There is, however, little to be gained by mere hypotheses, and it will be more satisfactory to consider the facts that favour a chemical explanation of the process.

Essentially the question to be proved is, whether neutral toxin-antitoxin mixtures (*i.e.*. such as have become innocuous to test animals) can once more, under given con-

ditions, develop toxic properties, a result which would be impossible if the toxin had been destroyed. In this connection, Buchner found that a mixture of tetanus toxin and tetanus antitoxin, barely innocuous to mice, remained toxic for guinea-pigs.

These results appear in a different light from our present standpoint, and the interpretation to be placed upon them leads me to anticipate a subsequent section of this paper. In such a mixture which approaches the Lo dose certain derivatives of the toxin are present in a free condition, viz.: toxoids and toxones—derivatives that exercise a toxic action on the one species of animals, but not on the other (mice). Similar effects have been noted by different observers in animals characterised as "hypersensitive." I refrain, however, from a discussion of this hypersensitive condition, as the subject still remains a most obscure one.

It would be rash to draw any conclusions of a general character from the uncertain, incomplete and confused observations that exist.

Now, oddly enough, it is just the investigations of Martin and Cherry which are considered the chief proof against the destruction theory, which originated with Roux, that make the matter appear doubtful. Roux and Calmette showed that whilst snake-venom is resistent to heat, the corresponding antitoxin is not. When a mixture of the two components, neutral to animals, was exposed to heat, they found that the mixture became toxic once more. The English authors have, however, shown that the effect as observed by Roux, only occurred when the mixture was heated shortly after its preparation—such a mixture is of itself toxic, and therefore contains unchanged toxin. If one allows the mixture to stand for about twenty minutes at room temperature, it becomes non-toxic and remains so, even after being subjected to heat. With this observation before us the hypothesis of Roux that both components exist unchanged in the neutral mixture becomes so untenable that even a supporter of the destruction theory might accept the same as valuable corroborative material. From this short summary it will appear that the reasons generally brought

forward in favour of a chemical combination of toxin and antitoxin are not in a position to withstand criticism. On the other hand, one can in another way—viz.: by the methods of quantitative analysis—readily furnish the proof that the processes occurring in the neutralisation of toxin and antitoxin are of a quantitative character, and that one is dealing with a process following the laws of definite proportion, which can be illustrated by the example of the formation of a simple salt.

If ten equivalents of an acid are mixed with ten equivalents of a base (or 100 with 100), a neutral mixture is obtained in each instance. We find exactly the same relationship to exist in the interaction of toxin and antitoxin. If a given quantity of diphtheria antitoxin (*e.g.*, the immunising unit used as a test basis) is mixed with varying quantities of a given toxin, an amount of the toxin which is exactly neutralised by the I.U. can always be determined. To this dose of toxin I apply the term Lo dose (Limes O). Through the mixture of all the toxic groups with antitoxin, prepared by adding one Lo dose to one I.U., a neutral mixture results which has no injurious effect on test animals.

It has thus been proved that corresponding to the laws of definite proportion, by means of one such single estimation upon any given amount of serum, the corresponding Lo dose of the toxin can be determined by a simple process of multiplication.

I found, for example, on using $\frac{1}{10}$ I.U. the Lo dose of a given toxin to be 0·24. On making an analogous determination with 1 I.U. the Lo dose was found to be 2·4—exactly tenfold. Similar figures confirming the above results are to be found in the papers of Kanthack and Madsen.

A second important test result, however, behaves quite differently in this respect, viz., that which I have termed the L+ dose (Limes, death). The L+ dose is that amount of a given diphtheria toxin which is not completely neutralised by a given amount of diphtheria antitoxin (I generally use one I.U.), to the extent that *exactly* one toxin dose, sufficient to produce the death of a guinea-pig of 250 grm. weight, remains unneutralised. This toxin dose may be

termed one lethal guinea-pig equivalent. To give an example in the case of a given toxin: I found on using $\frac{1}{10}$ I.U. an L+ dose of 0·037, but on using one I.U. only the value 0·26 was obtained. We see, therefore, that the postulated law regarding multiplication of doses is only admissible for the Lo dose, but *not* for the L+ dose. An example will make clear the reason for this difference. If ten equivalents of HCl are mixed with eleven equivalents of KOH, one equivalent of KOH remains unneutralised. If, however, the same amount of alkali is to remain free on using 100 equivalents of acid, one must add *not* 10×11, but only 101 equivalents of acid. In the above mentioned example in which I mixed $\frac{1}{10}$ I.U. with 0·037 toxin there remained exactly one lethal guinea-pig toxin equivalent free. On mixing 1 I.U. with 0·37 toxin the resultant mixture contained ten free lethal guinea-pig equivalents, *i.e.*, a fatal dose for ten guinea-pigs. Therefore the value 0·37 for the L+ dose obtained by multiplication, but experimentally found to be 0·26, is much too large, inasmuch as the L+ dose on neutralisation with the corresponding amount of serum leaves only one lethal guinea-pig equivalent free.

However simple it may be to determine the questions affecting the Lo and L+ dose on one and the same toxin, the matter assumed quite a different aspect when, proceeding from the above data, I undertook a comparative examination of different toxins. Instead of finding the postulated uniformity, in accordance with the law of equivalents, there occurred such differences and variations that I gave up, for a long time, any hope of finding the right clue. The great importance pertaining to these questions leads me to give a brief outline of the method by which I was able to reach the goal. The diphtheria toxin contains, besides the specific secretory products of the bacillus, a large number of different substances (salts, meat extractives, peptone, &c.). These substances are, however, without any significance with respect to the specific action of the bacilli, inasmuch as they can neither produce the specific intoxication nor combine with the antitoxin. We may therefore, in our considerations, ignore completely these associated bodies, and, in a physio-

logical sense, regard the diphtheria poison as a solution of the specific toxin in an indifferent fluid.

The problem resolves itself into this:—Can we regard the toxin-broth as a solution of a *pure* substance, and as containing solely a body characterised by its toxic and combining properties? The method for deciding this question is a simple one, and consists in a comparative examination of different toxins. Suppose, in the case of a given weak poison, the fatal dose for a guinea-pig is 0·5 c.c., in the case of a second 0·03, and in the case of a third 0·002. Granting that the toxin solutions were pure, in the above-defined sense, it follows that in these different quantities exactly the same amount of toxin must be present, that is to say, one lethal guinea-pig toxin equivalent—on this assumption, the same amount of antitoxin ought to suffice to render each of the three doses innocuous. But this is not what is found to occur, as a matter of fact, inasmuch as each of these doses may require a differing amount of antitoxin for neutralisation.

On the assumption of a pure and definite toxin, a uniform amount of antitoxin (*e.g.*, 1 I.U.) ought, in the case of different toxin-broths, to neutralise such amounts that by means of the same, an equal number of guinea-pigs are always killed; or, in other words, the Lo dose of different poisons, as ascertained with 1 I.U., ought to possess exactly the same toxicity. I have carried out such investigations with great exactitude upon about twenty different toxins, and have found the most marked variations, so that in one case the Lo dose (reckoned for 1 I.U.) corresponded to sixteen, and in another extreme instance, to 136 lethal guinea-pig doses.

It follows from these facts that the toxic broth does not represent a pure toxin solution, in the above-defined sense, but that in addition to the poison it must contain other bodies, which, whilst capable of combining with antitoxin, do not possess any marked toxicity. At the same time these "non-poisons" (ungifte) are in so far specific, that each one only occurs in that nutrient soil in which the given micro-organism has vegetated.

The observations upon the so-called spontaneous attenuation of the toxin furnish a certain explanation of the origin and significance of such bodies. It is well known that a toxin-broth, in course of time, gradually diminishes in toxicity. A freshly prepared toxin-broth, having a lethal dose for guinea-pigs of 0·2, may, after some months, require several such doses to produce the lethal effect.

I have now definitely proved (and this has been confirmed by Madsen's excellent researches) that in the course of this attenuation the neutralisation power does not necessarily undergo the slightest modification, *i.e.*, the Lo dose of the freshly prepared toxin is exactly the same as that of the attenuated.

How is this phenomenon to be explained? If a toxin broth, when freshly made, possesses the same Lo dose (in relation to 1 I.U.) as it does after attenuation, it follows that in both phases it must contain exactly the same amount of groups capable of combination, groups which may be represented by toxin or by the combining "non-poison" (ungift).

When, therefore, we find in the case of a given poison that the Lo dose remains the same in both phases, but that whilst at first it represented fifty, and finally only twenty-five lethal doses, it necessarily follows that these several twenty-five toxin equivalents have become transformed quantitatively into twenty-five equivalents of a "non-poison" capable of combination. This quantitative change points to the combining "non-poison" being a derivative of the toxin. Corresponding to this genesis I designate as toxoids such species of combining "non-poisons."

But it is specially to be noted that other species of combining "non-poisons" also occur in the toxin broth, which are primary secretory products of the diphtheria bacillus, and which, therefore, I distinguish by the special name of toxones, in order to distinguish them clearly from the toxoids.

The toxones possess less affinity for the antitoxin than toxins and toxoids, so that in the course of the partial neutralisation of a poison, the latter become neutralised

before the former. Through this circumstance it is easy to estimate with approximate accuracy the amount of the toxone, as I have already shown in my earlier researches upon the standardisation of the diphtheria toxin. I may mention that the amount of toxone present can be readily estimated by the difference between the Lo and L+ doses —the greater this is the greater the amount of toxone present. On testing a number of fresh toxins in which the toxone was still unimpaired, I found that the L+ dose was double that of the Lo dose. In these cases the poison contained exactly the same amount of toxone as toxin, so that the diphtheria bacillus had simultaneously produced with each part of toxin an equal amount of toxone.

As regards the properties of the toxone I will only mention here that it does not possess the property of killing test animals acutely, but it possess a certain power of producing indurations at the seat of injection, which are, however, less marked than those due to the toxin. On the other hand—and this would allot to the toxone a most important *rôle*—it appears to produce the slowly developing diphtheritic paralyses, as my own observations and those of Madsen and Woodhead tend to show. The circumstance that not only in the diphtheria patient, but also in animals infected with living bacilli such paralyses occur, favours the view that the toxone represents a primary secretory product of the diphtheria bacillus.

As a result of these various observations the question suggested itself whether the toxoids represent uniform substances (Einheitliche Stoffe), or whether they also consist of several groups. I was able to prove that in old diphtheria toxin-fluids at least three different species of toxoids occur which are differentiated through their affinity to antitoxin, and which I distinguish as proto-deutero- and trito-toxoid. Amongst these the proto-toxoid has the maximum affinity to antitoxin, and becomes neutralised in the first instance and before the other toxoids by antitoxin. I cannot now refer in detail to the lines I was led to follow, and I must therefore refer the reader to my publication upon the constitution of the diphtheria toxin and to the very detailed and clear ex-

perimental researches of Madsen, which fully confirm my observations. The guiding principle which led me to the determination of the actual constitution of the diphtheria poison and its varying composition of toxin and toxoid is a relatively simple one, and depends upon the *partial* neutralisation of a constant toxin amount with varying amounts of antitoxin. With this object in view suppose that one has determined (*e.g.*, with one I.U.) the Lo dose of a poison which is represented by x c.c., and which contains, say, 100 toxin doses—this toxin amount x is mixed with fractions of 1 I.U., say, $\frac{1}{5}$, $\frac{2}{5}$, $\frac{3}{5}$, $\frac{4}{5}$, I.U., and for each of these mixtures the number of lethal doses that still remain is determined.

In the first mixture, prepared with the smallest amount of antitoxin, only those portions of the poison (toxin and toxoid) become neutralised which possess the greatest affinity to antitoxin, whilst corresponding to the increasing additions made of antitoxin, the weaker and the weakest components eventually become neutralised, and of these the toxones, possessing as they do the least affinity, combine last of all. If we represent such a process of neutralisation in a simple schematic fashion, and assume that the broth contains only one kind of poison and one kind of toxoid, in exactly the same amounts, the experiment may assume three phases, which are dependent on the toxoid becoming neutralised—(1) simultaneously, or (2) subsequently to the toxin. The following table, in which the lethal capacity of the individual mixtures is given, will illustrate this point :—

	Lo (= 100 toxin doses).		
	I.	II.	III.
$\frac{1}{5}$ I.U. ...	100	80	60
$\frac{2}{5}$,, ...	100	60	20
$\frac{3}{5}$,, ...	80	40	0
$\frac{4}{5}$,, ...	40	20	0
$\frac{5}{5}$,, ...	0	0	0

It will be evident that, on this simple supposition, a single estimation carried out on any given mixture gives one a full insight into the constitution of a toxin. If we find, on mixing with $\frac{3}{5}$ I.U., the value 60, we are un-

doubtedly dealing with a toxoid of the same affinity as the toxin.

In the case of the poisons of more complex constitution, obviously one analysis is not enough; four to six are generally necessary for the adequate analysis of these toxins. I refrain from discussing the numerous analyses of individual toxins I have carried out, or the method of graphically illustrating the results in the form of the "toxin-spectrum." I will only touch on a few of the results of these prolonged investigations. It was found that the immunising unit used as a basis for official testing purposes contains 200 combining units, the combining unit being represented by that amount of antitoxin which is exactly sufficient to neutralise the lethal dose for a 250 grm. guinea-pig, *i.e.*, one lethal guinea-pig toxin equivalent.

The same equivalence with respect to the antitoxin can also be exercised by the *non*-toxic constituents of the broth, *i.e.*, the toxoids and toxones. If one ever succeeded by chemical methods in obtaining a diphtheria toxin, free from toxoids and toxones, its constitution would be such that (tested with 1 I.U.) the Lo dose would be sufficient to kill exactly 200 guinea-pigs.

Such an ideal toxin, according to my observations, is not capable of existence, as the bacilli always produce toxone along with the toxin, and consequently the number of lethal doses contained in the Lo dose is always considerably less than 200. On the other hand I have succeeded, and Madsen likewise, in finding two toxins which partially contain such a pure poison. In the case of the toxin I investigated, if one added to the Lo dose (which *per se* kills eighty-four guinea-pigs) $\frac{100}{200} = \frac{1}{2}$ I.U., the toxicity of the mixture was found to be 23. But if one added to the Lo dose a larger amount of antitoxin, viz., $\frac{115}{200}$ I.U., the mixture then possessed only a toxicity of 7. The further addition of $\frac{15}{200}$ I.U. (corresponding to fifteen combining units) had thus lessened the toxicity from 23 to 7, *i.e.*, it was diminished sixteen times. Consequently, through the addition of fifteen combining units of antitoxin, sixteen lethal guinea-pig doses had become neutralised, *i.e.*, each com-

bining unit had almost exactly neutralised *one* lethal guinea-pig dose. Thus the original mixture of the Lo dose + $\frac{1}{2}$ I.U. was so constituted, that of the portions remaining unneutralised the pure toxin possessed the maximum affinity and became neutralised in the first instance.

If the symbol T represent toxin, there became neutralised under these conditions—

T T T T T

Such pure toxin "zones" in the toxin spectrum are very rare and are only met with in the case of freshly prepared toxins. In contrast to this, one finds in the case of all toxins another form of combination represented by less extended "zones" of hemitoxin. One can with every toxin without exception prepare mixtures with antitoxin, which are so constituted that the addition of each additional combining unit ($\frac{1}{200}$ I.U.) reduces the toxicity by $\frac{1}{2}$ (in contradistinction to 1 in the case of pure toxin). There are always exactly the same portions of toxin and toxoid simultaneously neutralised corresponding to the following figure, in which T = toxin and I = toxoid, viz. :—

T I T I T I T

It requires no further demonstration that this occurrence can only be due to the affinity of the given toxin in question being the same as that of the toxoid. The question forces itself upon one, through what primary conditions is the toxoid formation brought about? The following observations will help towards an answer.

The toxin is defined through two attributes, of which the one is represented by the specific toxicity, the other by the specific combining power with antitoxin. I have already in the investigation upon the standardisation of toxins touched upon the question whether these two functions are bound up in the *same* complex of atoms, or whether the toxin molecule contains two independent groups, of which the one (the toxophore) conditions the toxicity, and the other (the haptophore) the combining property. I had at that time to leave the question undecided, but as the result of my further investigations upon toxoids I am now in a position to decide this question in the sense of duality.

The evidence in favour of the complete separation of the two functions rests especially upon the discovery of the hemitoxins, which, as mentioned above, are to be met with in all toxin broths to a greater or less extent. In the case of hemitoxin, there are always simultaneously neutralised equal parts of toxin and toxoid. Both these bodies must, therefore, possess exactly the same affinity to antitoxin. If we consider that the hemitoxin is derived from pure toxin through the half of the toxin molecule changing into the toxoid molecule, it follows that in the course of the metamorphosis of toxin into toxoid the affinity to antitoxin does not undergo the slightest modification but remains exactly the same. This is only comprehensible on the supposition of the existence of two different groups which are completely independent of one another.

We must, therefore, thus represent the action of the poison that with the help of the haptophore groups, the toxin molecule becomes "anchored" to the cell, and that it comes in this way within the sphere of action of the toxophore group. The poisoning is an effect of the toxophore, the antitoxin production an effect of the haptophore group.

The physiological significance of these two groups is of very different character, as will be seen from what has just been said. The biological rank of the two groups of the toxin molecule does not appear to be equivalent. The toxophore group, with functions of a ferment-like character, must as regards constitution be more complicated than the haptophore group, whose simple functions might well correspond to a simple structure.

This point of view would explain the ready disintegration of the toxophore group and the relative stability of the haptophore group. We thus see that the supposition of two different groupings explains, in the easiest manner, the phenomena observed in the attenuation of the diphtheria toxin, and especially the fact that the combining power of the broth remains intact, whilst the toxicity markedly sinks. From this point of view some phenomena observed, in the case of certain toxins, may be explained in an unconstrained fashion. The singular fact has been noted by Courmont

that frogs, treated with large doses of tetanus toxin, will remain for an indefinite period without toxic symptoms, if they are kept in a cool atmosphere. If, however, the frogs are placed in an incubator, a quickly fatal tetanus intoxication immediately ensues. Dr. Morgenroth proved, in my Institute, that the healthy animals, under cool conditions, had already fixed the tetanus poison in their central nervous system. If he gave to the frogs a sufficient quantity of tetanus poison and injected, after they had remained some time in a cool place, an amount of tetanus serum more than sufficient to neutralise the entire toxin dose, supposing the toxin to be still harboured in the blood, there yet occurred, despite the serum treatment, a typical and quickly fatal tetanus so soon as the animals were placed in the incubator.

This was, of course, only done after some days in order to allow sufficient time for the absorption of the serum. In the case of the cold frogs a portion of the tetanus poison must have left the blood and passed into the tissues, as only under this supposition can the absence of the serum action be understood. The experiments made by Dr. Billinger, and confirmed by Professor Dönitz, are probably to be explained in the same way, that marmots during their winter sleep are not affected by tetanus toxin, and first succumb to its action when they wake out of the same. We must, therefore, suppose that the *haptophore* groups of the tetanus toxin can become fixed by the nervous system at the lower temperature, but that the toxophore groups first become active above certain temperature limits. Such a thermic limitation to the activity of the toxophore group may at first appear remarkable. But numerous analogies are to be found amongst a class of bodies closely related to the toxins —the ferments. For example, any given quantity of rennet ferment when kept at zero produces no casein precipitation, but this immediately occurs when the milk in question is placed at a higher temperature. Apparently we have here a complete analogy to the frog experiments.

A second important question can be readily explained on the supposition of the existence of two groups, viz.: the question of the so-called incubation period observed in the

case of most toxins with very few exceptions (snake venom). When on the injection of a single fatal dose of tetanus toxin the first symptoms appear twenty-four, forty-eight, or fifty-two hours, the question arises whether we are here dealing with a function of the toxophore or haptophore group. This point has been decided for diphtheria and tetanus toxin by Prof. Dönitz by the intravenous injection of a given quantity of toxin and the estimation of the amount of serum necessary, when directly administered, to completely neutralise the toxin effect. This dose was so conditioned that it completely neutralised the toxin present in the blood. If, however, a little time was allowed to elapse between the injection of toxin and antitoxin (six minutes) it was found that the single neutralising dose was no longer adequate to prevent death. A portion of the toxin must therefore have left the vascular system and a dose sufficient to produce death been fixed by the central nervous system. It follows that the union between poison and tissue, which is a function of the haptophore group, occurs quickly. Further, the long incubation period must be referred to a slow functionating of the toxophore groups. Whilst in the case of the ordinary bacterial poisons the latent period does not generally last longer than twenty-four hours, there are substances which show a much longer latent period, *e.g.*, the toxones which produce the diphtheritic paralyses in test animals after two or three weeks.

The dual groups found in the case of the toxins occur also in the case of ferments. Anti-ferments can be produced in the body which completely neutralise the action of the given ferment. Dr. Morgenroth by successive injections of rennet ferment produced a serum which neutralised large quantities of the rennet ferment *in vitro*.

As already stated, the haptophore group of the toxin is relatively stable, the toxophore or ferment-like group is unstable, and readily undergoes disintegration. With these facts before us the formation of hemitoxin can be readily explained. Taking the above material and collective observations as a basis, we may reconstruct the origin and fate of the hemitoxins in the following manner:—The process usually

occurs in two phases, the hemitoxin is derived from pure toxin, and the toxoid from hemitoxin, *e.g.* :—

T T T T T T

T I T I T I

I I I I I I

The toxin is thus not a uniform entity, but contains two modifications which are present in exactly the same amount. The one modification readily disintegrates and forms hemitoxin, the other modification on disintegration forms pure toxoid. We may confidently state that the difference between the two modifications does not depend upon the haptophore group, as these possess the same combining power in toxoids as in toxin. The destruction of the toxin action points more emphatically to the toxophore group, and we must assume that the toxin contains two different toxophore groups, and that these two varieties are always simultaneously produced. The recent investigations of E. Fischer help towards an understanding of this phenomenon. These investigations show that as regards the enzymes and their objects of attack a similarity in molecular configuration must exist if a reaction is to occur. Fischer has rendered it most probable that the ferment group, analogous in many respects to the toxophore group, possess an asymmetrical constitution. If this is so *a priori* two possibilities exist—either the diphtheria bacilli produce one single group, or they produce simultaneously two. When one cell simultaneously produces two asymmetrical components, it frequently occurs that the two components are formed in exactly the same amount, *e.g.*, inactive oxalic acid. On such a supposition the formation of hemitoxin is capable of an unconstrained explanation. In many instances with the aid of micro-organisms an optically active half of a racemic compound has been split off, and one and the same organism may at one time attack the levo- and at another the dextro-rotatory modification of the compound.

E. Fischer has noted similar differences in the action of ferments.

The above considerations might help one to understand how in the toxophore group the one modification is first

attacked on storage of the broth and its original toxicity becomes reduced by one half (hemitoxin). It is difficult to decide what special influences control this change. It may be due to the presence of slowly-acting enzymes, which gradually develop their activity under certain conditions that arise in the conserved broth.

ON A NEW PATHOGENIC STREPTOTHRIX.

BY GEORGE DEAN, M.B.

FROM a review of the recent literature on the subject of Actinomyces and of the Steptotricheæ[1] one is driven to the conclusion that "actinomyces" is a term used to include several different species of micro-organisms. Hitherto, there has been a tendency to accept the histological demonstration in the tissues or discharges, of club-shaped organisms with a rosette arrangement, as sufficient proof that the pathological condition under consideration was due to the presence of one definite organism, the "actinomyces"; but recent researches have demonstrated that any such inference is unsafe.

The existence of filamentous forms with club-shaped swellings on their ends, arranged in rosette fashion, has been shown by several observers to belong to widely different organisms.

Thus, Professor Max Gruber[2] has described an organism, the "Micromyces Hofmanni," discovered as an accidental

[1] Since this paper was sent in for publication, a number of communications relating to this subject have appeared. Thus, Lubarsch, "Zur Kentniss der Strahlenpilze," and Schulze, "Über die Strahlenpilzformen des Tuberculoseerregers," *Zeitschr. f. Hygiene u. Infectionskrankheiten*, Bd. xxxi., Ht. 1, 1899, have shown that a number of organisms, *e.g.*, the Streptothrix Eppingeri, the Timothy Grass bacillus, etc., when introduced into experimental animals by the methods suggested by Friedrich and Babes or by direct inoculation into the kidney give ray fungus forms. Levy, "Über die Actinomyces gruppe (Actinomyceten) und die ihr verwandten Bakterien," and Bruns, "Zur Morphologie des Actinomyces," *Centralblatt f. Bacteriologie*, Bd. xxvi., No. 1. The latter describes a form of Actinomyces with certain peculiar features. It was altogether non-pathogenic to experimental animals.

[2] Gruber. "Über eine neue Eiterung erregende Microbenart. Micromyces Hofmanni." *Transactions of the Seventh International Congress of Hygiene and Demography.* London, 1891. Vol. ii., p. 65.

contamination, which possessed this characteristic. This microbe was found to be pyogenic in experimental animals, and gives rise, in the lesions produced by it, to the formation of beautiful filaments with club-shaped swellings arranged in rosettes. This organism is quite distinct from any form of actinomyces.

That the tubercle bacillus can present a ray-fungus form in the tissues has been shown by several workers.

Friedrich,[1] by injecting an emulsion of tubercle bacilli into the left ventricle through the carotid artery, has obtained a peculiar type of miliary tuberculosis. In the nodules there can be demonstrated by a special method of staining, "die bacillen inmitten eines schönen Kranzes strahlig angeordneten und so gestalteten Keulen oder Kolben wie wir sie als für Actinomyces characteristisch anzusehen pflegen."

Babes and Levaditi[2] have obtained similar results by injecting an emulsion of an attenuated culture of tubercle bacilli into the meninges and brain of rabbits. A month after inoculation the bacilli are found in masses of filaments having the size and appearance of the granules of actinomycosis. The preparations may be stained in various ways, but good results are obtained by Gram's method and orcein, the filaments retaining the methyl violet and the rosette of club-shaped processes taking on the orcein stain. Their illustrations represent, as they call it, a veritable "tuberculose actinomycotique."

To pass from forms which are obviously only remotely related to actinomyces, it is clear from the descriptions that the organisms obtained from actinomycosis by some of the chief workers at this subject are distinct varieties if not separate species. Thus, Wolff and Israel's[3]

[1] Friedrich. "Uber Strahlenpilzähnliche Wuchsformen des Tuberkelbacillus im Thierkörper." *Deutsche Med. Wochenschrift*, xxiii. 1897. S. 653.

[2] Babes et Levaditi. "Sur la forme actinomycosique du bacille de la tuberculose." *Arch. de Méd. expér.*, ix. 1897. 1041.

[3] Wolff und J. Israel. "Ueber Erzeugung mittelst Culturen des Strahlenpilzes." *Berliner Klin. Wochenschr.* No. xiii., S. 309. 1890. "Über Reincultur des Actinomyces und seine Übertragbarkeit auf Thiere." *Virchow's Archiv.* 125 Heft 1., S. 11. 1891.

streptothrix differs in a number of characteristics from that of Boström.[1] Among other differentiating features of the streptothrix Israeli, may be mentioned its superior anaerobic growth, the persistence of bacillary forms in agar cultivations in place of the dichotomously dividing filaments of Boström's organism, and the positive inoculation experiments on animals.

Sauvageau and Radais[2] hold that it is not necessary to assume, as suggested by Boström, that Wolff and Israel were using impure cultures. They state, however, that they consider comparative experiments with the two organisms are necessary.

Kruse,[3] who accepts Wolff and Israel's streptothrix as a cause of actinomycosis, holds that independent confirmation of their results is desirable.

Jurinka[4] states that the cultures obtained by him were identical with those of Wolff and Israel, but the fact that his inoculation experiments were always negative does not tend to confirm this idea.

Kischensky's[5] streptothrix in many points appears to resemble Wolff and Israel's, but inoculation experiments are not described. Kischensky also succeeded in growing his organism on gelatine, which Wolff and Israel failed to do.

Van Niessen,[6] in a recent paper in *Virchow's Archiv*, still doubts the purity of Wolff and Israel's cultures, and refuses to accept the conversion, in experimental animals, of the bacillary form into filaments with club-shaped processes. He appears to the writer to take altogether too narrow a view of the subject.

[1] Boström. "Untersuchungen über die Actinomykose des Menschen." *Ziegler's Beiträge*, ix., Heft 1. 1890.

[2] Savaugeau et Radais. "Sur les genres Cladothrix, Streptothrix Actinomyces et description de deux Streptoth. nouveaux." *Annales de l'Institut Pasteur*, vi., p. 242.

[3] Kruse. *Die Mikroorganismen.* Flügge iii., Auflage, 1896. Zweiter Theil, S. 57.

[4] Jurinka. "Zur Konservativen Behandlung der menschlichen Aktinomykose." *Mitteilungen aus den Grenzgebieten der Medizin und Chirurgie*, I. Bd., 2 Heft, S. 139. 1896.

[5] Kischensky. "Über Aktinomycesreinculturen." *Archiv für experimentelle Pathologie und Pharmokologie*, xxvi., S. 79, 1889.

[6] V. Niessen. "Die Aktinomyces-Reinkultur." *Virchow's Archiv*, Bd. CL., S. 482, 1898.

Gasperini[1] holds that actinomycosis in the ox is due to different varieties of actinomyces, approaching each other at certain points of their cycle of development, but perfectly distinct at others. He states that certain of these which do not permit of cultivation on artificial media do so after passage through the dog. This is of some interest in relation to the organism described in the present paper which may have been modified in its passage through the horse.

Eppinger[2] is of opinion that there are probably many species of actinomyces, and regrets that sufficient attention has not been given to the morphological and biological characters of the group. This view is also supported by Unna,[3] who suggests that the clinical types may vary according to the variety of organism present.

It would be undesirable and, indeed, impossible to enter here into a complete review of the literature on the subject of the pathogenic streptotricheæ. A short account, however, of the recent work on the subject is necessary in relation to the present communication.

Of those streptotricheæ which in the animal body produce granules, consisting of filaments with club-shaped endings arranged in rosette fashion, the organisms described by various writers can be seen to belong in the main to two groups, the first closely resembling or identical with Böstrom's organism, the second, evidently a smaller group, corresponding with Wolff and Israel's. Other organisms with the characteristics mentioned have, however, strongly marked features of their own, and must be regarded as distinct species, *e.g.*, the streptothrix maduræ of Vincent.[4]

In addition to these there is a great group of streptotricheæ which do not possess in the animal body club-shaped swellings on the ends of the filaments. This group

[1] Gasperini. "Versuche über das Genus Actinomyces. XI. Internat. Med. Kong. Rom." *Ref. Centralblatt für Bakteriologie und Parasitenkunde*, Bd. xv., S. 684.

[2] Eppinger. *Die Durch Cladotricheen* (Streptotricheen, etc.) *hervorgerufenen Erkrankungen. Ergebnisse der Allg. Pathol. Lubarsch u Ostertag*, 3 jahrg., S. 328, 1897.

[3] Unna. "Aktinomykose und Madurafuss." *Deutsche Med. Zeitung*, No. 6, 1897.

[4] Vincent. Etude sur le Parasite du "Pied de Madura." *Annales de l'Institut Pasteur*, 1894, p. 129.

contains the well-known organisms described by Rosenbach,[1] Eppinger,[2] Schmorl,[3] Nocard,[4] Rabe[5] and others of less pathological significance.

To this literature there have been several recent additions indicating a hitherto unsuspected importance of the group as disease-producers.

Garten[6] has described a streptothrix which he isolated from the body of a young man, who for two years had suffered from necrosis of the vertebræ and ribs with abscesses, empyema and resulting sinus formation. In the pus were found filaments, long and short rods and cocci. There was no appearance of swellings on the ends of the filaments, of clubs, or of rosettes.

He obtained cultivations on all the ordinary media. From the cultures he got branching filaments dividing into coccal forms without an intermediate bacillary stage. He describes the cocci as having the power of slow movement. In only three animals—two rabbits and one guinea-pig—out of thirty-seven inoculation experiments were positive results obtained.

Sabrazès[7] and Rivière describe two cases in which streptotricheæ were found. The first was the case of a young man who had abscesses in the brain, right lung and right kidney. Rivière observed filaments arranged in radiating fashion and with rectangular branches, in the brain and kidney abscesses, but failed to find those in the lung abscess.

[1] Rosenbach. "Über das Erysipeloid." *Archiv f. klin. Chirurgie*, xxxvi., 346.

[2] Eppinger. "Über eine neue Pathogene Cladothrix und eine durch sie hervorgerufene Pseudotuberculose (Cladotrichica)." *Ziegler's Beiträge*, ix., 287.

[3] Schmorl. "Über ein pathogenes Fadenbacterium." (Streptothrix Cuniculi.) *Deut. Zeitsch. f. Thiermed.*, xvii., S. 375, 1891.

[4] Nocard. "Note sur la maladie des bœufs de la Guadeloupe, connue sous le nom de Farcin.' *Ann. de l'Institut Pasteur*, 1888.

[5] Rabe. "Über einen neuentdeckten pathogenen Mikroorganismus beim Hunde." *Berliner thierärztl. Wochenschr.*, 1888.

[6] Garten. "Uber einen beim Menschen chronische Entzündung erregenden, pleomorphes Mikrobion." *Deutsche. Zeitschr. f. Chirurgie*, Bd. xli., Hft. 4 u. 5, S. 257.

[7] Sabrazès and Rivière. "Sur un Streptothrix rencontré dans un cas d'abscès du cerveau et à un infarctus supuré du rein." *La Presse Médicale*, 1894, Sept. 22. Also "Les parasites du genre Streptothrix dans la pathologie humaine." *Mercredi Médical*, 1895, pp. 485 and 486.

Cultivation on gelatine, potato and bouillon gave only cocci under aerobic conditions, but anaerobically there developed white, cloudy colonies of the streptothrix. The organism stained by Gram's method. A guinea-pig died of coccus infection, but no other animal experiments are mentioned. The second streptothrix of these authors was obtained from the sputum and from a subcutaneous abscess in pure culture. It was aerobic, grew on and liquefied gelatine and produced a yellow pigment. It was not pathogenic to experimental animals unless injected along with lactic acid.

Ferré and Faquet[1] isolated in pure culture a streptothrix from a cerebral abscess in an epileptic. It grew well on different media. Both in the pus and from potato cultures it had the form of filaments with knobbed ends staining by Gram's method. It was not pathogenic for guinea-pigs, but, inoculated under the dura mater of rabbits, could be found throughout the whole body without having produced any observable reactive change.

Bucholz[2] has described a case of lung disease running a fairly acute course, which he attributes to a streptothrix infection. The clinical picture was one of tuberculous pneumonia and empyema.

The lungs presented the appearance of an advanced tuberculosis chiefly affecting the lower lobes. There were present patches of consolidated tissue which appeared to be necrotic. These had evidently originated from areas of lobular infiltration and had indurated and atelectic areas surrounding them. There were also cavities containing fœtid pus and communicating with an empyema. These resembled tubercular vomicæ, but had more ragged walls.

The microscopic sections of the consolidated areas gave the appearance rather of a fibrinous than of a tuberculous pneumonia.

Examination of the necrotic areas of the parts round the

[1] Ferré and Faquet. "Streptothrix et abscès du cerveau." *Mercredi Médical*, 1895, p. 441.

[2] Bucholz. "Über menschenpathogene Streptothrix, ein Beitrag zur Ætiologie des acuten Lungenzerfalls." *Zeitschrift für Hygiene und Infectionskrankheiten*, xxiv., S. 470, 1897.

cavities by Gram's method showed a thick network of delicate branching threads of the thickness of tubercle bacilli. The filaments showed, sometimes complete, sometimes interrupted staining, and here and there a thickening, but there were no coccal or bacillary forms.

In the atelectic regions masses of streptococci were present, but not in the infiltrated and necrotic parts. Neither before death nor at the sectio could tubercle bacilli be found.

Bucholz believes that the streptothrix was the cause of the lobular pneumonia, with the resulting necrosis, and that the streptococcus invasion was secondary.

Cultivations failed owing to the overgrowth of the plates by a variety of proteus. The author explains that the find in the lung was the first indication of the great interest of the case.

Scheele and Petruschky[1] in a difficult case with multiple skin abscesses, pleuro-pneumonia, cystitis and pyelitis, in which the diagnosis was thought to lie between glanders and influenza with septicæmia, found in the sputum streptothrix forms. The organism was present in the abscesses in pure culture and grew on gelatine and glycerine-agar, but not, like allied species, on potato or glucose-agar. This is the first time in which a streptothrix infection has been diagnosed during life.

Petruschky[2] briefly referred to a second case, in which a similar organism was isolated from the sputum. It differed from the previous one in giving a more abundant growth on, and in causing liquefaction of gelatine.

These cases were brought forward at the fifteenth and sixteenth meetings of the International Medical Congress, and the full reports and descriptions have not yet appeared.

Rullmann[3] has described a case from V. Ziemmsen's

[1] Scheele and Petruschky. "Verhand. des XV. Congress für Innere Medicin." Ref. *Deutsche Med. Wochenschr. Vereins-Beilage*, No. 17, S. 124, 1897.

[2] Petruschky. *Deutsche Med. Wochenschr. Vereins-Beilage* xvi., No. 13, S. 78, 1898.

[3] Rullmann. "Uber eine aus sputum isolirte pathogene Streptothrix *Münchener Med. Wochenschr*, No. 29, S. 919, 1898.

clinic in which, associated with a localised lesion near the root of the right lung, probably of a bronchial gland, there was present in the sputum a streptothrix. It occurred as tough masses composed of interlacing filaments in pure culture. A few of the filaments showed branching. They stained well, though irregularly, with the ordinary bacterial stains, and retained the colour with Gram's method. The organism grew well on all the ordinary culture media.

On Loeffler's blood serum it produced bullet-shaped colonies which had a chrome yellow colour. Bouillon culture had a good surface growth and became turbid. On solidified egg albumin, aerobically it produced filaments, anaerobically rods with club-shaped ends like diphtheria bacilli.

Inoculation in animals, mice, guinea-pigs, rabbits, gave positive results whether the organism was introduced into the subcutaneous tissues, the peritoneal cavity, the trachea, or the circulation.

In the lesions the filaments were present and could be re-cultivated. Suppuration of the lymphatic glands was a striking feature of the infection. A horse which was inoculated appeared to be ill for some time and had a cough, but recovered. Four and a half months after, it was killed, and showed in the lungs and liver a few hard nodules, but in these no streptothrix could be found.

This organism is chiefly characterised by the formation of a chrome yellow pigment on blood serum, and by the fact that only filamentous forms were present in the experimental animals.

Flexner[1] has described a streptothrix infection of the lung closely resembling that in Bucholz's case, in the clinical history, in the morbid anatomy, and in the microscopical character of the organism found in the affected tissues.

The clinical signs and symptoms pointed to a case of pulmonary tuberculosis.

At the *post-mortem* the lungs were found to be partially consolidated, whole lobes being affected. In these areas

[1] Flexner. "Pseudo-Tuberculosis Hominis Streptotricha." *The Journal of Experimental Medicine*, vol. iii., Nos. 4 and 5, 1898.

there was beginning softening (disintegration with early cavity formation). The rest of the lung tissue was congested and œdematous, and contained, scattered through it, caseous areas and calcified nodules. Nodules resembling tubercles and usually translucent were scattered over the peritoneal surfaces, the omentum being especially affected. The mesenteric glands were not enlarged, but on section opaque tubercles were visible in them. Histological examination of the lung showed a cellular exudate, intra- and inter-alveolar, the cells being mostly polynuclear leucocytes. The process of disintegration was extensive, and was characterised by the presence of an increased number of nuclear fragments and irregularly staining detritus. In certain regions the exudate was largely fibrinous. Circumscribed nodules having the size, form, and appearance of miliary tubercles were present in small numbers. They were chiefly composed of epithelioid cells, no giant cells being found. These were present, however, in the peritoneal nodules, which in their structure closely resembled tubercles. The bacteriological examination revealed the presence of an organism scattered through the affected areas in the form of branching filaments often in convoluted masses, readily staining by Weigert's method. The branches were sometimes capped at the extremity by a conical swelling. The filaments sometimes stained irregularly, giving a beaded appearance and rarely showed a tendency to break into short rods.

In the peritoneal nodules the organism took more the form of rods with a marked tendency to beading. The microbe was about the thickness of B. Proteus. No tubercle bacilli could be found in any of the lesions.

Attempts at cultivation of the streptothrix failed. A guinea-pig injected with a suspension from the consolidated lungs showed no local re-action or enlargement of glands, but wasted and died after nine weeks. *Post mortem* no lesion was found. This experiment is strongly against the view that the process was a genuine tuberculosis, notwithstanding its great resemblance to that condition.

Notwithstanding the fact that the term "pseudo" has

been so widely condemned it appears inevitable that it should constantly recur in connection with pathological conditions. It has been used in two ways in connection with streptothrix infection, certain cases having been described as pseudo-tuberculosis, as in Flexner's case, others having had the term pseudo-actinomycosis applied to them, as in some of the cases to which reference is about to be made. Sawtschenko[1], in a case which had the clinical features of actinomycosis, found in the pus, granules resembling actinomycotic granules, but which on examination proved to be zooglœa masses of bacilli.

The organism did not stain by Gram's method. It was an obligatory anaerobe, growing on liquid blood serum, glycerin-bouillon and glycerine-agar at body temperature. It coagulated blood serum without forming an acid. On admission of O it formed long filaments without branches.

A similar organism also not staining by Gram's method and not forming branches on cultivation has been described by Dor[2] from an actinomycosis of the lower jaw. This organism grew on liquid media only, and was aerobic.

Berestnew[3] has also described a case of this nature.

The patient was a child who had the symptoms of broncho-pneumonia. Later there developed an intrathoracic abscess, which was found, on resection of the ribs, to communicate with a cavity in the lung. The child died with symptoms of pyæmia. Along with other lesions there were found patches of broncho-pneumonia, cavities in the lung, and a cerebral abscess. In the pus of the brain abscess, and in the broncho-pneumonic foci at the apices of both lungs, was found only one organism which occurred in granular masses. It took the form of long straight slender rods, not staining by Gram's method. The bacillus proved to be anaerobic, and grew on sugar-agar, and in bouillon mixed with hydrocele fluid.

[1] Sawtschenko. "Über Bacilläre Pseudo Actinomykose" (Russisch). *Russisches Archiv f. Pathologie*, Bd. i., S. 184, 1896. Ref. *Baumgarten's Jahres, bericht*, 1896, S. 613.

[2] Dor. "Une nouvelle mycose à grains jaunes; ses rapports avec l'actinomycose; est-ce une variété de l'actinomycose ou une espèce différente? *Gazette Hebdom. de Méd. et Chir.*, No. 47, 1896.

[3] Berestnew. Über Pseudoaktinomycose." *Zeitschr. f. Hygiene und Infectionskrankheiten*, Bd. xxix., Ht. 1, S. 94, 1898.

In liquid media, in addition to rods similar to those found in the pus, it grew in the form of long filaments. It was not pathogenic to rabbits, guinea-pigs, or dogs. It produced in the peritoneum of mice at the site of inoculation foci of suppuration with cachexia and death in about two weeks. In the sections of the affected tissues of the child masses of the organism with radiating filaments at the periphery were found.

These cases, described by Sawtschenko, Dor, and Berestnew, contribute a most interesting addition to our knowledge of pathogenic agents. If their existence is confirmed, they form a group worthy of much wider study.

The other three organisms described by Berestnew[1] were all from cases which would be regarded clinically as typical actinomycosis.

The first was a sub-maxillary abscess, the second an empyæma, and the third a lung actinomycosis. Filaments, clubs, and rosettes could be demonstrated in the discharge from all these.

Berestnew succeeded in cultivating from these cases two microbes which differed essentially from actinomyces, and had also characters which differentiate them from any hitherto described form of streptothrix. The organisms obtained from the first two cases were identical except that the first preferred anaerobic conditions.

It grew on agar and solidified blood-serum in the form of opaque white angular vegetations, which sent processes into the depth of the medium. In bouillon it grew as opaque nodules at the bottom of the test tube, the growth after a week reaching the size of a hemp seed. In this medium it produced an unpleasant odour. No growth could be obtained on potato or gelatine. It died out after two generations.

The organisms from the cultures were found to be mostly rods of very various sizes and shapes, which were often club-shaped, and showed beading. Filaments were also present, which were for the most part unbranched. It was non-pathogenic to mice, rabbits, and guinea-pigs.

[1] Berestnew, *loc. cit.*

The other organism from this group was characterised by producing an intense yellow pigment on agar and by a slow growth on gelatine without liquefaction of the medium. The growth on bouillon in this case occurred in the form of minute particles adhering to the side of the test tube. It also took a bacillary form on artificial media.

In rabbits and guinea-pigs it produced slow-growing infiltrations when injected subcutaneously. On intraperitoneal injection no effect was produced.

The former of these two microbes described by Berestnew resembles in several features the streptothrix about to be described. There are, however, several points of difference which will be discussed in a separate section.

Source and Isolation of the Organism.

The streptothrix was obtained from a horse which was undergoing diphtheria immunisation. Before admission to the stables at The Poplars it was seen by Mr. Villar, F.R.C.V.S., who applied the mallein test with a negative result.

Some time after the animal was discovered accidentally to have a hard nodule below and near the angle of the jaw. The swelling was about the size of a walnut, and appeared to be an enlarged indurated sub-maxillary gland. The animal seemed to be in excellent health, but, as a precautionary measure, it was again tested with mallein and also with tuberculin, in both cases no reaction being obtained.

The nodule remained apparently unaltered for about three months, and was then observed to be increasing in size. In a week or two from this time it reached the size of a goose's egg, and distinct fluctuation could be made out.

The skin over the swelling was shaved and disinfected, and a sterile hypodermic needle introduced. The pus obtained in this way was mixed with neutral beef tea, and from it a dozen agar, agar-glycerine, and blood-serum tube cultures were made. I then freely opened and scraped the abscess cavity with a Volkmann's spoon.

The pus which escaped was pale yellow in colour, and the material evacuated resembled the contents of a caseating tuberculous gland. In the pus no granules could be observed.

A number of cover glass preparations were made and examined for tubercle bacilli, but none could be found.

In preparations stained by Kühne's methylene blue or Gram's method there were observed, fairly uniformly distributed throughout the preparations, delicate filaments which suggested actinomyces. No granules consisting of interlacing filaments, nor clubs nor rosettes could be found.

The filaments stained irregularly, giving a beaded appearance. They showed true dichotomous branching. *See* fig. 1.

In addition to the microscopic examination, and the preparation of cultures, some of the pus was at once inserted into subcutaneous pockets in the thighs of two guinea-pigs, with the view of ascertaining whether tubercle bacilli were present or not. The result was negative in regard to tubercle. The details will be found in the section on the animal experiments.

The Cultivation of the Organism.

On the twelve tubes three organisms grew, two bacilli of no interest in regard to the case, and the streptothrix, of which only two isolated colonies could be found, one on the surface, the other in the depth of an agar tube.

From these two colonies subcultures were made on various media.

The organism does not grow with great freedom on solid media. Sometimes only one out of six, and often only one out of three tubes of the same media, inoculated from the same culture, develop colonies. The organism tends to die out if it is kept on solid media for several generations, and I have found it advisable to grow it alternately on solid and in liquid media containing blood serum or ascitic fluid.

This difficulty of cultivating and keeping alive certain species of the streptotricheæ on artificial media has been observed by several writers.

Thus Berestnew[1], and Mosselman and Liénaux[2] describe species which can be cultivated only in bouillon. Mosselmann and Liénaux's organism could not be kept alive beyond one month.

Agar (surface culture).—The colonies appear from the third to the fifth day as opaque white points. Generally from the outset these have irregular outlines, with angular projections both along the surface of the medium and into its depth. The processes tend to form secondary branches, and in this way a fully-grown colony a fortnight to three weeks old presents an appearance like a little mass of white coral, which is very characteristic. *See* fig. 4.

When the surface of the medium is broken in making the inoculation, the growth into its depth is sometimes much more extensive than the surface growth, and presents the same coralline appearance. *See* fig. 3. Sometimes the colony departs from this type, and has a fairly uniform unbroken margin. The maximum size is about $\frac{3}{16}$ inch in diameter.

The colour is an opaque white, with a somewhat wax-like lustre. No change of colour ever takes place in the growth.

Agar (*puncture culture*).—The organism grows well till a depth of about $\frac{3}{4}$ in. from the surface is reached; below this the colonies become very small.

Agar glycerine.—Cultures identical with agar culture.

Agar alkaline (5 c.c. normal NaHO per litre after neutralisation).—Growth somewhat more free than on the above-mentioned media. The appearances are identical.

Glucose agar.—No growth could be obtained.

Bouillon neutral and alkaline (v. alkaline agar).—In these two the results are similar. The growth takes place only at the bottom of the tube, never on the surface or through the medium. The broth appears unaltered. In this case the growth takes the form of a little mass, which, after a month, has reached the size of a pea, with numerous subdividing processes, so that it looks exactly like a minia-

[1] Berestnew, *loc. cit.*

[2] Mosselman and Liénaux. "L'actinomycose et son agent infectieux," *Ann. de Méd. Vétér.* (Brux.), Août, 1889, p. 409.

ture cauliflower or a piece of white coral. Even if, when inoculating the tube, several small pieces of material are introduced, these tend to adhere to one another.

The mass has considerable cohesion and can be shaken about without breaking. No change in the reaction of the medium can be ascertained.

Yeast medium, as suggested by Spronck,[1] for the diphtheria bacillus, proves an excellent pabulum for the growth of the streptothrix. In this there is less tendency than in bouillon to form one coherent mass. The growth takes the form of a mass of irregularly-branched flocculent particles at the bottom of the tube.

Bouillon with ½ per cent. formate of soda gave a good growth, similar to the last.

Maltose bouillon also proved a favourable medium. Various mixtures of ascitic fluid and normal horse serum (Marmorek's media) with bouillon were tried, and proved themselves very favourable to the growth of the organism.

Sometimes in these it developed like a little semi-transparent mass of textile material with opaque points on it.

Infusions of straw, oats and hay, with (1) their natural reaction; (2) neutralised; (3) alkalinised, all gave rather poor results.

Milk.—No growth or change in the milk.

Solidified blood serum.—No growth occurred on the medium, and, as this seemed a remarkable result, it was repeated a number of times and controlled by growing diphtheria bacillus, actinomyces, the first generation of the tubercle bacillus, &c., on the same medium, but always with the same result.

Egg albumin gave no growth either when the egg was inoculated by boring the shell or when the albumin was drawn into sterile Pasteur pipettes.

Potato.—(1) with its natural acid reaction; (2) alkalinised; (3) glycerinated. All gave no growth.

Gelatine gave no growth.

[1] Spronck. "Préparation de la toxine diphthérique; suppression de l'emploi de la viande." *Annales de l'Institut Pasteur*, p. 701, 1898.

The most suitable temperature for the organism was from 35 to 37° C.

The organism grew best under aerobic conditions; anaerobically by Buchner's method with pyrogallic acid the growth was very poor.

The Microscopical Appearance of the Organism from different Cultures.

From agar cultures.—Preparations from such cultures were made at intervals from the fourth day till six weeks after inoculation. The appearances did not vary greatly during that time. The organism is seen to be extremely pleomorphic. The great majority of the microbes are in the form of rods, which vary from short, plump rods to long rods about the size of the large form of the diphtheria bacillus. The average may be taken as about 3 to 5 μ in length, ·3 to ·4 μ in breadth.

The rods are often bent, and even spirillum forms may be seen. Club-shaped, battledore and drumstick forms are present, though not in great numbers.

Many of the rods stain irregularly by Gram's method and by Kühne's or Loeffler's methylene-blue and by dilute carbol-fuchsin, thus presenting a beaded appearance. The chromatin particles vary from two to five in number.

The forms of the bacilli and the polar staining make the preparations closely resemble those of the diphtheria bacillus; in fact, in many fields of the microscope it could not be distinguished from preparations of the large type of that organism. The bacilli stained by Gram's method or with dilute Ziehl-Nielsen's solution looked larger than when stained by methylene-blue. A very few short filaments, which are thicker than the rods, are present, but the picture presented is totally different from what one sees in the case of the ordinary laboratory actinomyces (Boström's), where in a six-days old preparation the whole field is occupied by a branching network of filaments. Compare figs. 6 and 7 with fig. 2.

In addition to the form mentioned, a few round, oval, or

ellipsoidal bodies are to be found. These bodies have a diameter of about ·8 to 1 μ. Some of them appear to me to be developed on the ends of straight rod forms, others in the middle of the rod. These bodies are of frequent occurrence in this group of organisms and have been regarded by some as a coccus form, by others as a type of spore.

From bouillon, formate bouillon, straw and yeast media.—The preparations are practically the same as from agar. The rod form is still predominant, though a few short filaments may be found. *See* fig. 5.

From ascitic fluid or serum bouillon.—Branching filaments are present in greater numbers in this medium. The filaments resemble those from an agar preparation of ordinary actinomyces, but the branching is much less marked.

The filaments stain very irregularly both by Kühne's methylene-blue and by Gram's method. Segments of the filaments, often sharply demarcated, and with rounded ends, retain the methyl violet, whereas the adjoining part may be decolourised. By staining by Gram's method and then by Ziehl-Nielsen's carbol-fuchsin mixed with equal parts of water, one gets very beautiful double staining.

Unlike the bacilli belonging to the tubercle group, this organism, when stained by carbol-fuchsin, shows no resistance to the action of decolourising agents, such as the mineral acids or alcohol.

Animal Experiments.

Rabbits.

It may be stated at the outset that the rabbits used for these experiments were half-grown animals from 700 to 1,000 grammes weight.

Rabbit 1.—This animal received an intraperitoneal injection of an emulsion from an agar (alkaline) culture of the second generation. The animal suffered a slight loss of weight, and died at the end of a month.

The *post-mortem* examination revealed the presence of

five or six nodules situated between the layers of the great omentum. They formed a grape-like cluster, varied in size, from a pin's head to a hemp seed, and were of a pale yellow colour. Microscopic examination showed the various forms of the organism, including rosettes. A more minute description of the structure will be given under a separate section, to avoid repetition under each experiment.

Rabbit 2.—An emulsion from a culture of the second generation on agar glycerine was injected intraperitoneally. This animal died after ten days.

At the site of inoculation was a small plaque the size of a threepenny piece, and scattered round this within an area of two inches diameter were five or six nodules from the size of a hemp-seed to a pea. These nodules were similar in appearance to those in the previous experiment, but were less firm in consistence.

Rabbit 3 received 2 c.c. intraperitoneally of an emulsion in bouillon from a fourteen-days-old culture on neutral agar of the third generation.

The animal showed no symptoms; it was killed in fifty-nine days. A nodule the size of a pea, very firm and sharply demarcated, lay at the hilum of the spleen, and invaded its substance for an eighth of an inch.

Rabbit 4.—In this experiment 2 c.c. of a culture in ascitic fluid bouillon of the second generation were injected intraperitoneally. At the site of inoculation after a week there could be felt a nodule the size of a horse bean. The animal was killed after twelve days.

The nodule on dissection was found lying outside the peritoneum and projecting through it. It was of the size mentioned, with two or three projections as large as hemp-seed, and a nodule similar in size and appearance to these latter was situated about one inch from the main mass. The nodules were pale yellow, and were surrounded for about $\frac{1}{8}$ in. by a hyperæmic zone.

On section of this nodule a thick caseous purulent looking material could be scraped out containing coccal, bacillary, and rosette forms, and from this material successful pure cultivations were made.

Rabbit 5.—This animal was injected at the same time, and in the same manner as in the previous experiment. It was killed after twenty-eight days. The appearances differed little from those found in the previous experiment; the nodules were rather larger and firmer. *See* fig. 11. Cultivation succeeded in this experiment also.

A series of intravenous injections was carried out parallel to the above five intraperitoneal injections.

Rabbit 6 received intravenously 2 c.c. of an emulsion from the same alkaline agar culture as rabbit 1. It appeared to be unaffected, and on its being killed after one month no lesion could be discovered, and cultivations from the blood and viscera gave negative results.

Rabbit 7 received intravenously 2 c.c. of an emulsion from the same alkaline agar culture as rabbit 2. It died after twelve days, but no lesion could be discovered, and cultivations were negative.

Rabbits 8 *and* 9 received intravenously 3 c.c. of ascitic fluid bouillon culture, and in both cases the results were negative, the animals being killed and nothing found.

A series of three rabbits was inoculated from a twenty-days-old culture in bouillon containing ½ per cent. formate of soda in a closed flask, with the view of ascertaining whether the partially anaerobic conditions so obtained affected the virulence of this organism, as was suggested by Gasperini in the case of certain races of actinomyces.

Rabbit 10 received 2 c.c. of this culture intravenously and 2 c.c. in the subcutaneous tissues of the ear.

An intense inflammatory reaction took place at the site of inoculation and a nodule the size of a hemp seed with a halo of injected vessels developed in the ear, persisting till the death of the animal, which took place twenty-two days after the inoculation. Beyond this nodule, which had the usual structure, no further lesion could be found and cultivation experiments gave negative results.

Rabbit 11 received 3 c.c. intravenously from the same culture. The animal died in twenty-four days. No lesion was visible and cultivation experiments were negative.

Rabbit 12 received 2 c.c. from the same culture intra-

peritoneally. Died on the twenty-second day. Examination gave negative results.

These three experiments will be referred to in the summary of the animal experiments.

In the next series of experiments an older cultivation in formate broth was taken, viz., one of two months' growth.

Rabbit 13 received 3 c.c. of this culture intraperitoneally. The animal died nine days after inoculation. On the peritoneal surface of the cæcum was found an elevated plaque about ¾ in. by ½ in. It was pale yellow, with a zone of injected vessels round it. The margin was serrated, small radiating projections extending from it. Towards the centre of the plaque were several points about ⅛ in. diameter, of a paler yellow colour than the rest of the formation.

Rabbit 14.—3 c.c. of the culture were injected intra-abdominally. The point of the needle punctured the kidney. The animal died in seven days.

Between the kidney on its anterior surface and the peritoneum an area of adhesion, about 1 in. in length, pale yellow and fibrinous looking.

The cortex of the kidney in this region had several rounded and triangular areas, the size of a millet seed, and of a pale yellow colour. They were very sharply demarcated from a hyperæmic region round them. The microscopic examination showed that these were necrotic areas, containing large numbers of the bacillary and filamentous forms. A few of these with branches could be found, but no distinct club or rosette formation. The organism extended into the tissues for some distance round and about the necrotic area.

Rabbit 15.—The third rabbit of this series received 3 c.c. of the culture intraperitoneally. It appeared to be unaffected and was killed two months after the inoculation. There were three or four nodules present in the omentum and mesentery, the size of a hemp seed, and of very firm consistence. The organism was easily demonstrated in the sections of these nodules.

Rabbits 16 *and* 17.—Each received intraperitoneally 2 c.c. of an emulsion in bouillon, from an alkaline agar culture of the fourth generation. Both animals appeared unaffected

and were killed after two months. No lesion could be found.

Experiments on Guinea-pigs.

The experiments on these animals were much less successful than those on rabbits. As before mentioned, with the view of ascertaining whether there were tubercle bacilli present in the original pus, a small quantity of this was inserted into subcutaneous pockets in the thighs of two guinea-pigs.

Guinea-pig 1.—In this animal there was a slight swelling for a day or two at the site of inoculation. A week later a firm nodule the size of a hemp seed could be felt. A fortnight after inoculation the animal was killed, and the nodule examined. In it no tubercle bacilli were found, but a small clump of interlacing filaments radiating at the periphery, but without any well marked club formation.

Guinea-pig 2.—Inoculated at the same time and in the same way as No. 1. Beyond a slight induration at the site of inoculation nothing was observed in this animal. It remained in perfect health for two months, when it was killed. No tuberculous or other lesion was found.

Guinea-pigs 3 *and* 4.—Each received intraperitoneally 2 c.c. of a bouillon emulsion of an agar culture of the second generation. The animals remained unaffected and were killed in six weeks. No lesion found at section.

Guinea-pigs 5 *and* 6 had 2 c.c. of a formate broth culture injected subcutaneously in abdominal wall. Both gave negative results.

Guinea-pigs 7 *and* 8.—Each received subcutaneously 2 c.c. of culture of the second generation in ascitic fluid bouillon.

No. 7 developed a swelling the size of a hazel nut at the site of inoculation. This after a fortnight burst, and discharged a thin pale yellow pus which contained streptococcus pyogenes and bacillus coli communis, along with the streptothrix in filamentous form.

No. 8 gave a negative result.

Experiments on Mice.

Two out of four mice inoculated died, one in three weeks, the other in five weeks, after inoculation, but in neither could any lesion be found.

Experiments on Pigeons.

Two pigeons were inoculated. One received subcutaneously over the pectoral muscle 2 c.c. of formate broth culture of the third generation. Result negative.

The other received in the same way 2 c.c. of an ascitic bouillon culture of the third generation. This animal developed a swelling the size of a pea at the site of inoculation. It was killed in twenty-four days. The bulk of the swelling appeared to consist of old blood clot and fibrin, but round it were three nodules the size of millet seed, pale yellow and very firm. In the sections of these the streptothrix was present in convoluted masses of the filamentous form. No rosette structure.

Summary of Animal Experiments.

The most successful inoculation experiments were those on rabbits. Out of seventeen rabbits nine gave some naked-eye lesion due to the presence of the organism.

The guinea-pig proved more resistant; only two out of eight gave lesions, and one of these was due to a mixed infection. Of the mice, two animals out of four died, but showed no naked-eye lesion. In one pigeon, out of two inoculated, nodules were produced.

The organism could be obtained in pure form from the lesions twelve and twenty-eight days after inoculation. It was not attempted in every case to recultivate the organism, as this entailed a great amount of labour, a large number of tubes requiring to be made for each case, but in every case a single cultivation was made to exclude the presence of other organisms, such as the pyogenic cocci. This result was confirmed in every case by the microscopical examination of the lesions.

The method that gave the largest number of positive results was intraperitoneal injection; eight out of eleven rabbits were so infected. In a number of these a subcutaneous lesion also was produced at the site of inoculation.

Intravenous injection either failed to give any result or the animal died without any observable lesion, perhaps from some toxic effect. This happened in three out of six experiments.

Affanassiew and Schultz,[1] with a streptothrix isolated by them, got similar results with old cultures, probably from some toxic substance contained in them.

The most important facts pointing to a toxic action were obtained by me in the case of three rabbits which were inoculated from a flask in which the streptothrix had been grown for twenty days in bouillon containing ½ per cent. formate of soda. These animals died with cachectic symptoms in twenty, twenty-two and twenty-four days after inoculation, and no lesion to account for death could be found. (Formate broth alone does not produce any such effect.) This may be a coincidence, but it was remarkable that the three animals should die in so nearly the same time and without any other apparent cause to explain the circumstance.

In other cases filtered bouillon cultures produced in the ears of rabbits a marked local inflammatory reaction with formation of vesicles.

Structure of the Lesions in the Experimental Animals.

The nodules and affected tissues were fixed and hardened either in alcohol or in Zenker's solution and then embedded in paraffin in the usual manner.

The ordinary histological stains were employed in examining the structure. For demonstrating the organism in the tissues the best results were got by Gram's method (Weigert's modification), alone or combined with picro-

[1] Affanassiew and Schultz. III. Med. Cong. Russ., ref. *Baumgarten's Jahresbericht*, Bd. v., S. 398.

carmine or eosine. Very beautiful results were obtained in this manner.

The Erhlich-Biondi stain and Plaut's fuchsin and picric acid method both gave excellent results.

Examination of Lesion in Early Stages, say, Seven Days after Inoculation.

The organisms in this case still retain the bacillary form. Branching filaments are present, but are not numerous. The organisms are surrounded by masses of fibrin, hæmorrhages and groups of polynuclear leucocytes. There is an active proliferation of fibroblasts in the neighbouring connective tissues. This stage is represented in fig. 8, a photomicrograph from a preparation stained by Gram's method and eosin. The blood corpuscles and polynuclear leucocytes are only faintly outlined in the photograph.

Tissues such as the kidney, into which the organism has found its way, are in a necrotic condition (*see* rabbit 14). The cells in these areas are blurred and stain badly. The necrotic tissue has sometimes a considerable amount of fibrin deposited in it, as shown by Weigert's method. The affected parts are surrounded by a hyperæmic zone. In and near the necrotic tissues the organism is present in considerable numbers.

Examination of Lesion at more Advanced Stage, from Twenty to Thirty Days after Inoculation.

At the periphery of the nodule there is a capsule consisting of young fibrous tissues, fibroblasts and spindle-cells. From this capsule a number of septa, with the same structures, pass into the nodule and subdivide it. The spaces thus formed contain a mass of cells, for the most part polynuclear leucocytes, with a few so-called "epithelioid" cells. Remains of hæmorrhages and traces of fibrin may still be present. Among these structures the organism is found in various stages of its development. The bacillary and coccal forms are still represented, though in much smaller numbers.

Filamentous forms are now predominant. They are most frequently arranged in dense, interlacing clumps, with the ends of a number of the filaments radiating from the periphery. This is the pre-rosette stage in which the ends of the filaments have not yet assumed the flask or club-shaped swelling. In other groups these have gone on to form well-marked clubs arranged in rosette fashion with a zoogloea-like mass in the centre. In comparing the clubs with those found in the tongue of the ox, one is struck by the frequency with which in the rabbit this organism gives rise not to a blunt but to a lanceolate club, the point of the spear-shaped process pointing outwards. I do not mean by this to assert that the organism is characterised by this form, but its frequency is certainly remarkable. *See* figs. 9 and 10, a photomicrograph from a section from rabbit stained by Gram's method.

The clubs when stained by Gram's method retain the methyl-violet well.

Gasperini[1] found in experimenting with actinomyces on a calf that the clubs developed in four days, but in one of my rabbits, which died on the seventh day, I could find only bacillary, coccal, and a few filamentous forms.

Examination of Lesions Two Months Old.

In nodules of this age there is a tendency to cavity formation in the centre of the nodule. This is surrounded and partly filled by badly-staining granular *débris*. Outside this the "epithelioid" cells are now more numerous.

A few giant-cells packed with fragments of filaments can in some sections be found near the margin of the cavity.

The organism is now almost entirely in the club-shaped or spear-shaped form. A few filaments with globular swellings in their course are present. There are to be seen in the nodule at this stage numerous spherical hyaline

[1] Gasperini. "Nuove ricerche sull' actinomicosi sperimentale." *Annali d'Igiene sperimentale*, vol. vi., p. 475.

bodies which stain deeply by Gram's method and also with carbol-fuchsin. These vary in size from minute points to about the size of a red-blood corpuscle.

The Differential Characters of the Streptothrix from those nearly allied to it.

The streptothrix resembles the micromyces Hoffmanni in giving no growth on potato, gelatine and blood serum, and in producing subcutaneous lesions in rabbits containing the rosette form, but differs from it in having the characteristic coral-like growth, especially in bouillon, instead of a powdery deposit, in producing no brown pigment, and in giving rise to distinct lesions when inoculated intraperitoneally, which the micromyces Hoffmanni fails to do.

The streptothrix resembles the streptothrix Israeli in having persistent bacillary forms on agar and in the positive inoculation experiments on animals, but differs from that organism in its characteristic coralline growth, especially in bouillon, by the absence of growth on egg albumin and blood serum, and markedly by a distinct preference for aerobic conditions, and by showing, under these, greater rapidity and freedom of growth.

The last feature alone is sufficient to differentiate the two organisms.

The streptothrix alba of Rossi-Doria (actinomyces bovis albus of Gasperini)[1] is readily distinguished, as it forms a membranous growth on gelatine with liquefaction of the medium.

Berestnew's[2] organism from his first two cases of pseudo-actinomycosis resembles in a number of features the organism we are considering, but its cultural characters, the fact that it died out so quickly and that it was not pathogenic to rabbits, guinea-pigs and mice, are sufficient to differentiate it.

Berestnew's third organism is distinguished by the production of a yellow pigment on agar, and by the occurrence of growth on gelatine. From the short review of the litera-

[1] Gasperini. "Versuche über das Genus Actinomyces," ref. *Centralblatt für Bakter.*, xv., S. 684.

[2] *Loc. cit.*

ture given it will be seen that the other forms recently described have features quite distinct from the present organism.

I have compared cultures on various media and microscopical preparations of the streptothrix with those of actinomyces obtained from three different sources.

(1) From Dr. Salter, cultivated from an appendicitis in the human subject.

(2) From the Jenner Institute at Chelsea, of unknown origin.

(3) From Kràl, Prague, from the human subject.

I found these three to be practically identical with one another, and to be widely different from the streptothrix. The growth in all of these is much more abundant and rapid; they all grow on potato and on gelatine with liquefaction of the medium, and on potato and agar they produce pigments, yellow and brown of various shades, and black. The microscopical characters of the growth have been already referred to. The organism has also been compared with all the members of the group which it has been possible to obtain, namely, streptothrix Nocardii, streptothrix Eppingeri, streptothrix nigra, streptothrix canina, but to none of these does it present any marked resemblance.

Conclusions.

(1) That the organism described in this paper produced in the horse a disease which might be described clinically as actinomycosis.

(2) That, resembling certain described forms, this streptothrix has characteristics which differentiate it from these.

(3) That it differs widely from the ordinary actinomyces of the laboratory.

(4) That the organism has a bacillary form which persists on agar, &c.

(5) That the bacillary form introduced into experimental animals gives rise to nodules, and in those are developed filaments with lanceolate and club-shaped processes arranged

in rosette fashion, thus confirming Wolff's and Israel's results with an allied organism.

(6) That the results obtained in this research tend to confirm the hypotheses (*a*) that the streptotricheæ are more nearly allied to the bacilli, such as the diphtheria and tubercle bacilli, than to the moulds; (*b*) that actinomycosis is a disease due not to one specific micro-organism, but to a number of allied species.

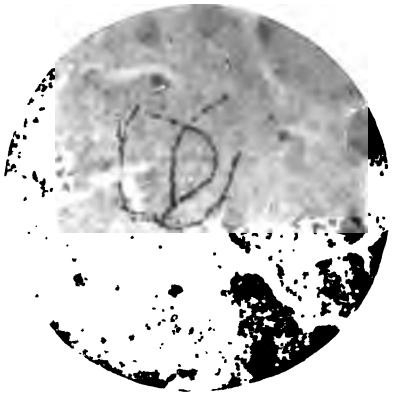

FIG. 1

FIG. 2

FIG. 3

FIG. 4

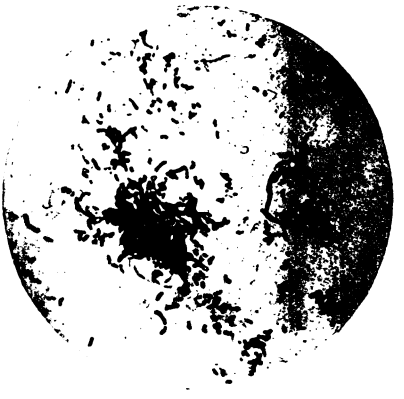

FIG. 5

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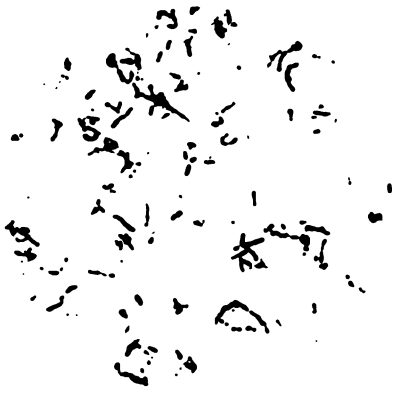


FIG. 6

FIG. 7

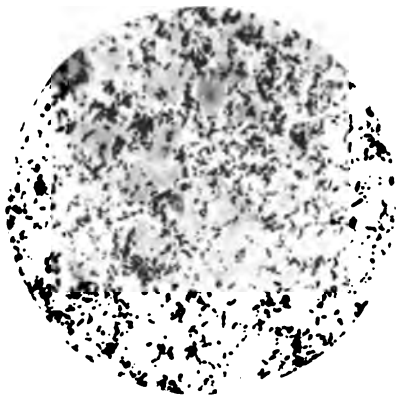


FIG. 8

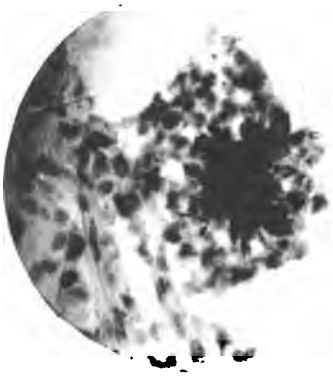


FIG. 9

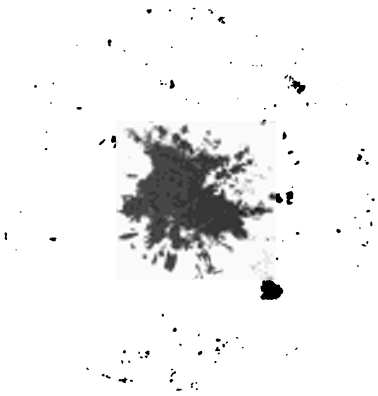


FIG. 10

Photoprint, Bale & Danielsson, Ltd.

FIG. 11

Photoprint, Bale & Danielsson, Ltd.

DESCRIPTION OF FIGURES.

Fig. 1.—Photomicrograph of cover-glass preparation of pus from submaxillary abscess in horse, stained by Gram's method. Shows branching and beading of filaments. × 1000.

Fig. 2.—Photomicrograph of cover-glass preparation from agar culture six days old of ordinary actinomyces. Stained by dilute carbol fuchsin. × 750.

Fig. 3.—Photograph of a colony of the streptothrix one month old in the depth of agar medium. Shows branching and coral-like appearance. Slightly magnified.

Fig. 4.—Photograph of surface colony of the streptothrix on agar, aged six weeks. Slightly magnified.

Fig. 5.—Photomicrograph of cover-glass preparation from culture in ascitic fluid bouillon, one month old. Shows a few branching filaments and many rods. × 750.

Fig. 6.—Photomicrograph of cover-glass preparation from agar culture of the streptothrix six days old. Stained by dilute carbol fuchsin. Shows beaded rods and a few short filaments. For comparison with fig. 2 and to show resemblance to large diphtheria bacilli. × 750.

Fig. 7.—Similar preparation from culture six weeks old, more highly magnified. × 1100.

Fig. 8.—Photomicrograph of cover-glass preparation from the seven days' old lesion near the kidney of rabbit 14. Shows the beaded rods lying among red blood corpuscles and polynuclear leucocytes. Stained by Gram's method and eosin. × 750.

Fig 9.—Photomicrograph of section through nodule in omentum of rabbit 15. Shows rosette arrangement of the organism with lanceolate processes at the margin of cavity, surrounded by polynuclear leucocytes and epithelioid cells. Stained by picro-carmine and Gram's method. × 350.

Fig. 10.—Photomicrograph of section through nodule in abdominal wall of rabbit 5. Shows lanceolate processes of a rosette. Stained by Gram's method. × 350.

Fig. 11.—Photograph of nodule on anterior abdominal wall of rabbit 5. Reduced in size.

A CONTRIBUTION TO THE STUDY OF DIPHTHERIA TOXIN.

BY WILLIAM BULLOCH, M.D.

In his monumental work, "Die Werthbemessung des Diphtherieheilserums," Ehrlich has drawn attention to the modifications which occur in diphtheria toxin, these modifications chiefly affecting the behaviour of the diphtheria bouillon towards antitoxin. This paper contains the remarkable discovery of the existence of a series of bodies (toxoids), which, like toxin, have an *affinity* for antitoxin, but which differ from toxin in their *valency* for antitoxin. In the main these toxoids belong to three types—protoxoid, in which valency is greater; syntoxoid in which it is equal; and epitoxoid (since called toxone), in which the valency for antitoxin is less than that of the toxin.

These discoveries show that the constitution of diphtheria bouillon is much more complex than was formerly believed, and the study of toxoids has become a prime necessity in the estimation of the antitoxic value of sera for clinical purposes. With the exception of the papers of Ehrlich and Madsen, the literature contains few contributions of value, as most writers have under-estimated the difficulties of standardising toxins and serums, with the result that many of the observations and deductions made, even in recent publications, are inaccurate.

The study of the constitution of the two diphtheria filtrates in this paper is offered as a contribution to this subject.

For the precise knowledge of the constitution of a diphtheria bouillon four factors are necessary.

(1) The exact determination of the "simple lethal dose" (in Ehrlich's sense). This involves the use of a very large

series of guinea-pigs of approximately the same weight, and the estimation of the results is often associated with considerable difficulty.

(2) The determination of the exact neutralisation limit (Lo) which is obtained with 1 unit of antitoxic serum.

(3) The determination of the death limit (L+), where in spite of 1 unit of serum the animal succumbs between the fourth and fifth day.

(4) The determination of the difference D between L+ and Lo.

The difficulties associated with this subject have not been minimised by the extreme condensation and brevity of Ehrlich's writings, and from the fact that some of his deductions are not apparent from the data given. Nowhere is this more apparent than in the fundamental determination of the absolute combining value of the unit, which he gives as 200. In a later publication (4) this has been given with clearness and exactitude, which materially lessens the difficulties, and up to the present time this number is apparently correct, although the future may show that the absolute value of the unit is a multiple of 200.

As the toxins examined in this paper have special reference to the existence of toxone, it is essential that the method by which the toxone is estimated should be clearly understood. As one unit of serum corresponds to 200 combining groups of diphtheria bouillon, the equivalent of one combining group BE (Bindungs Einheit) being the simple lethal dose for M^{250} it follows that—

$$\text{Lo} = 1 \text{ unit} + x \text{ toxoid} + y \text{ toxin} + z \text{ toxone} = 200.$$

And—

$$\text{Lo} = 1 \text{ unit} + (200 - z)\ (\text{toxoid-toxin}) + z \text{ toxone} = 200.$$

And—

$$1 \text{ toxone} = \frac{1}{200 - z} \text{ toxoid-toxin.}$$

And—

$$z \text{ toxone} = \frac{z}{200 - z} \text{ toxoid-toxin}$$

This contains—

$$\alpha \frac{z}{200 - z} \text{ toxin-units}$$

(α = number of toxin units in Lo)

$$L + L^{\alpha} - 1 = D - 1 = \beta.$$

Therefore—

$$\alpha \frac{z}{200 - z} = \beta.$$

From this the value of z (the amount of toxone) is easily determined:—

$$\beta (200 - z) = \alpha z.$$
$$200 \beta - \beta z = \alpha z.$$
$$200 \beta = \beta z + \alpha z.$$
$$200 \beta = z (\alpha + \beta).$$
$$z = \frac{200 \beta}{\alpha + \beta}$$

Toxin I.—Culture grown in 2 per cent. peptone bouillon for thirty days, filtered through a Chamberland bougie, and conserved with toluol.

DETERMINATION OF THE SIMPLE LETHAL DOSE.

Dose of Toxin.	Weight of Guinea-pig.	Result.
0·25 c.c.	265 grms.	Dead in 51 hours.
0·24 c.c.	257 ,,	,, ,, 50 ,,
0·23 c.c.	235 ,,	,, ,, 51 ,,
0·22 c.c.	232 ,,	,, ,, 5 days.
0·21 c.c.	284 ,,	,, ,, 4 ,,
0·20 c.c.	265 ,,	,, ,, 5 ,,
0·20 c.c.	250 ,,	,, ,, 6 ,,
0·18 c.c.	250 ,,	,, ,, 9 ,,
0·10 c.c.	245 ,,	,, ,, 7 ,,
0·09 c.c.	250 ,,	,, ,, 7 ,,
0·08 c.c.	238 ,,	,, ,, 8 ,,
0·075 c.c.	270 ,,	,, ,, 10 ,,
0·072 c.c.	265 ,,	,, ,, 10 ,,
0·070 c.c.	270 ,,	Large Infiltration: recovery.
0·066 c.c.	250 ,,	,, ,, ,,
0·055 c.c.	245 ,,	Infiltration: recovery.
0·050 c.c.	250 ,,	,, ,,

The simple lethal dose is represented by 0·2 c.c.

The determination of L+ and Lo was made with one-tenth unit corresponding to Ehrlich's older test ; the material used for the estimation being samples of Höchst serum 250 ×. (Where standard glycerine serum cannot be obtained, or only in small quantity, it will be found that the Höchst serum standardised by Ehrlich in Berlin affords a very accurate gauge.)

Dose of Serum (Höchst) and Toxin. Serum. Toxin.	Weight of Guinea-pig.	Result on Fourth Day.
·0004 c.c. + 1 c.c.	300 grammes	Dead in 96 hours.
·0004 c.c. + 0·8 c.c.	260 „	„ „ „
·0004 c.c. + 0·72 c.c.	245 „	„ 72 „
·0004 c.c. + 0·64 c.c.	250 „	„ 72 „
·0004 c.c. + 0·60 c.c.	250 „	„ 120 „
·0004 c.c. + 0·50 c.c.	260 „	Large infiltration
·0004 c.c. + 0·48 c.c.	250 „	„ „
·0004 c.c. + 0·40 c.c.	255 „	„ „
·0004 c.c. + 0·36 c.c.	250 „	„ „
·0004 c.c. + 0·34 c.c.	295 „	Medium „
·0004 c.c. + 0·32 c.c.	275 „	„ „
·0004 c.c. + 0·31 c.c.	265 „	Small „
·0004 c.c. + 0·30 c.c.	260 „	No „
·0004 c.c. + 0.28 c.c.	240 „	„ „

According to Ehrlich's older test 0·30 c.c. was evidently the test dose which with ·1 unit (contained in ·0004 c.c. Höchst serum) gave complete protection to a guinea-pig weighing 260 grammes, the L+ value being 0·60 c.c. That the sample of Höchst serum actually contained 250 units per c.c. was shown from the testing by means of a toxin standardised by Professor Salomonsen, as also by Ehrlich's

own standard toxin, which, with his usual generosity, he placed in considerable quantity at our disposal.

As ·20 c.c. of toxin I. was the simple lethal dose, and 0·6 c.c. corresponded to the L+ dose of the new test of Ehrlich and 0·30 the Lo dose, the graphic formula may be represented in the usual nomenclature as

DTN 0·05 (0·2 + M^{250}).
L+ 0·6 = 3 fatal doses.
Lo 0·3 = 1·5 ,, ,,
D 0·3 = 1·5 ,, ,,

From the formula—

$$z = \frac{200\ \beta}{\alpha + \beta}$$

we thus see that the amount of toxone (z) is—

$$\frac{200 \times 0{\cdot}5}{1{\cdot}5 + 0{\cdot}5} = \frac{100}{2} = 50$$

i.e., the toxin contained exactly 50 toxone.

As the Lo value contains $1\frac{1}{2}$ fatal doses, the increment is $\frac{1}{2}$ fatal dose ($1\frac{1}{2}$—1 = $\frac{1}{2}$), and it is contained in $\frac{1}{3}$ of the Lo value.

The constitution of the bouillon may thus be represented,

135 toxoid + 15 toxin + 50 toxone.

In regard to the nature of the toxoid, it evidently must be syntoxoid, and the relation between the toxin and syntoxoid is 1 to 9.

The test dose contains toxone and toxin in the proportion of 5 to 15.

If one add $\frac{1}{3}$ Lo the toxone is set free, but the L+ is not reached because there is no free toxin. In order to obtain this result it will be necessary to add $\frac{2}{3}$ Lo more, and then the value L+ is obtained with 1 toxin free, *i.e.*,

$$L+ = Lo + Lo\ \tfrac{1}{3} + Lo\ \tfrac{2}{3} = 2\ Lo.$$

or as was obtained experimentally in quantity

·6 = ·3 + ·1 + ·2 = 2 Lo, which was ·30 c.c.

Toxin II.—This poison was obtained from a culture grown for thirty days in 2 per cent. alkaline peptone bouillon, and it was preserved by toluol, and in a hydrogen atmosphere.

The simple fatal dose as determined upon five animals was found to be 0·02 c.c.

It was standardised by titration against Ehrlich's test glycerine serum, and Lo and L+ determined as follows :—

Serum.	Toxin.	Result.
1 unit	+ 1 c.c.	Dead in 48 hours.
„	+ 0·9 c.c.	„ 48 „
„	+ 0·85 c.c.	„ 72 „
„	+ 0·82 c.c.	„ 72 „
„	+ 0·82 c.c.	„ 72 „
„	+ 0·81 c.c.	„ 96 „
„	+ 0·81 c.c.	„ 96 „
„	+ 0·80 c.c.	„ 100 „

The determination of the Lo value, with one-tenth unit, gave :—

Serum.	Toxin.	Result on Fourth Day.
·1 unit	+ ·2 c.c.	Dead in 48 hours
„	+ ·1 c.c.	Very large infiltration
„	+ ·09 c.c.	„ „ „
„	+ ·08 c.c.	„ „ „
„	+ ·078 c.c.	Infiltration
„	+ ·075 c.c.	No infiltration. Slight on 1, 2, 3 days
„	+ ·070 c.c.	No infiltration

The results of these experiments gave, therefore, L+ as ·8 c.c. with 1 unit and Lo ·075 with ·1 unit.

After two months the L+ and Lo values were again determined more precisely in relation to 1 unit.

The extraordinary difference between ·78 and ·79 was startling, the dose ·79 c.c. killing the guinea-pig in five days

with a very large infiltration, while the animal which received ·78 remained unaffected throughout.

Serum.	Toxin.	Result.	
1 unit	+ 0·82 c.c.	Dead in 96 hours	
„	+ 0·82 c.c.	„ 96 „	
„	+ 0·81 c.c.	„ 96 „	
„	+ 0·81 c.c.	„ 96 „	
„	+ 0·81 c.c.	„ 72 „	
„	+ 0·80 c.c.	„ 98 „	
„	+ 0·80 c.c.	„ 100 „	
„	+ 0·80 c.c.	„ 96 „	
„	+ 0·79 c.c.	„ 120 „	large infiltration
„	+ 0·78 c.c.	No infiltration, increase of weight	

·79 or ·80 represented the L+ value.

The graphic representation of this toxin stands thus :—

$$\begin{array}{ll} \text{DTN} & \cdot 5\,(\cdot 02 + \text{M}^{250}) \\ \text{L+} & \cdot 8 = 40 \\ \text{Lo} & \cdot 78 = 39 \\ \hline \text{D} & \cdot 02 = 1 \end{array}$$

The fact that D is not more than 1 shows that toxin is completely absent from the diphtheria bouillon, a result which is approximated by only one of the toxins (no. X.) examined by Ehrlich (3).

If ·79 represent the L+ dose (and it is often difficult to determine within one point) the D value works out to ·5, *i.e.*, less than 1, a proof that the toxin contained protoxoid, and the constitution of the bouillon may be represented as

160 Protoxoid + 40 Toxin + 0 Epitoxoid.

This remarkable toxin was fully investigated by Professor Ehrlich several months later, and certain variations were found which were confirmed about the same time by Dr. George Dean and myself. There can be little doubt that in the course of some months the toxin had altered slightly,

and it was further suggested by Professor Ehrlich that a considerable difference might possibly exist between the Berlin guinea-pigs and the Sudbury animals, a point of very considerable importance in such investigations. The

Toxin Dose.	Result.
0·023 c.c.	Dead in 3 days
0·020 c.c.	,, 3 ,,
0·018 c.c.	,, 2 ,,
0·017 c.c.	,, 4 ,,
0·017 c.c.	,, 3 ,,
0·017 c.c.	,, 4 ,,
0·016 c.c.	,, 5 ,,
0·016 c.c.	,, 5 ,,
0·015 c.c.	,, 4 ,,
0·015 c.c.	,, 2 ,, (hypersensitive)
0·015 c.c.	,, 5 ,,
0·014 c.c.	,, 7 ,,
0·014 c.c.	,, 5 ,,
0·014 c.c.	,, 6 ,,
0·014 c.c.	,, 2 ,, (hypersensitive)
0·014 c.c.	,, 6 ,,
0·014 c.c.	,, 2 ,, (hypersensitive)
0·013 c.c.	,, 6 ,,
0·013 c.c.	,, 6 ,,
0·013 c.c.	,, 3 ,, (hypersensitive)
0·012 c.c.	,, 4 ,,
0·012 c.c.	,, 6 ,,
0·011 c.c.	Alive.
0·010 c.c.	Alive.

remarkable difference between the effects of ·78 c.c. and ·79 c.c. were, however, confirmed by Ehrlich, the setting free of the toxin at ·79 c.c. killing English guinea-pigs, whereas it produced only severe induration in the Berlin animals. The influence of the temperatures at which the animals are kept is probably of importance, as the higher L+ dose of ·86 obtained by Ehrlich was also obtained by Dean and myself during winter, but not in summer. The determination of the absolute toxicity is a point of great difficulty, and one of great importance. The difficulty arises in the varying susceptibility of different animals, and hence arises the necessity of using very large numbers in order to ascertain this important datum.

The experiments quoted in the table in the preceding page were supplied by Professor Ehrlich.

Determination of the absolute toxicity of toxin II.

The determination of the absolute toxicity by myself, after receiving Ehrlich's results, gave approximately the same, and demonstrated the fallacy of using too small a number of animals.

Toxin.	Result.
·020	Dead in 3 days
·019	„ 3 „
·018	„ 4 „
·0175	„ 4 „
·0170	„ 4 „
·0160	„ 3 „
·0160	„ 5 „
·0160	„ 4 „
·0150	„ 5 „
·0150	„ 6 „
·0140	„ 6 „

From these experiments it is evident that the formula of the toxin had become modified.

DTN ·66 (·015 + M^{250})

L+	·86	= 57·2
Lo	·78	= 52
D	·08	= 5·2

Here, granting that the first determinations were correct (and the experiments were performed on an extensive series of animals), one is compelled to assume that the epitoxoid had formed later in the bouillon, although this is denied by Ehrlich. The experiments in reference to toxin II. are quoted *in extenso* to show the absolute necessity of working on a very large series of animals for the purposes of standardising toxine. The slightest defect in technique (dilutions, injection, &c.) and the deductions obtained from the use of a few animals are bound to lead to error.

In conclusion, I beg to express my best thanks to Professor Ehrlich for his exceptional generosity, advice and help at all times, and to Dr. Thorvald Madsen, of Copenhagen, for the explanation of many points of difficulty in regard to the standardisation of toxins and antitoxins.

The most important points on the subjects under discussion will be found in the following papers.

(1) Ehrlich, Kossel und Wassermann. "Ueber Gewinnung und Verwendung des Diphtherieheilserums," *Deutsche Medicin. Wochenschrift*, 1894.

(2) Ehrlich und Wassermann. "Ueber die Gewinnung der Diphtherieantitoxine aus Blutserum und Milch immunisirter Thiere," *Zeitschrift für Hygiene*, 1894, xviii., p. 239.

(3) Ehrlich. "Die Werthbemessug des Diphtherieheilserums und deren theoretische Grundlagen." Jena, 1897.

(4) Ehrlich. "Ueber die Constitution des Diphtheriegiftes," *Deutsche Medicin. Wochenschrift*, 1898, No. 38.

(5) Madsen. "Experimentelle Undersögelser over Difterigiften, Kjöbenhavn," 1896.

(6) Madsen. "Ueber Messung der Stärke des antidiphtherischen Serums," *Zeitschrift für Hygiene*, 1897, xxiv., p. 425.

THE BACTERIOLOGY OF THE NORMAL CONJUNCTIVAL SAC, FROM A REPORT OF 200 CASES, AND ITS PRACTICAL BEARING ON THE UTILITY OF ANTISEPTICS IN OPHTHALMIC SURGERY.

BY ARNOLD LAWSON, F.R.C.S.ENG.

THE bacterial condition of the normal conjunctival sac is a matter of general interest to ophthalmologists, not only from the merely scientific standpoint of ascertaining the variety and number of the contained bacteria, but also from its practical bearing on the methods of ophthalmic surgery.

If an apology is needed for this paper, it lies in the fact that the bacterial condition of the normal conjunctival sac has never been thoroughly worked out in this country, and, although a considerable number of investigations have been made in other countries, there has been so much diversity of opinion expressed, that a further contribution may help to a definite and satisfactory conclusion on this important subject.

I will, firstly, give a brief summary of the work of others before proceeding to the results of my own observations.

(1) There is a general consensus of opinion that the normal conjunctival sac contains bacteria in the majority of cases.

(2) Opinions differ as to whether the normal conjunctiva is ever sterile. Leber considered that it was frequently sterile.

Franke's [1] researches found the healthy sac sterile in 28 per cent. of cases.

More recently Morax has expressed his belief that the

[1] VON GRAEFE'S *Arch. of Ophth.* Bd. xxxix. Abth. iii.

normal conjunctiva is never sterile, and in this he is supported by Randolph[1] in a very recent article upon the subject. The question largely turns upon the impossibility of testing the whole conjunctival surface, so that sterility in inoculated tubes cannot be held to afford complete proof of the sterility of the entire conjunctival sac.

(3) Most, if not all, of the later writers agree that the number of bacteria present in the healthy conjunctiva is small.

(4) As regards the pathogenic properties of the bacteria found, earlier observers agreed that the pyogenic cocci were frequently present. The latest researches go to shew, however, that the *staphylococcus epidermidis albus*, a comparatively inert form of *st. pyogenes albus*, very frequently replaces the latter microbe. Randolph[2] thinks that probably all bacteria found in the normal conjunctiva have but little, if any, pathogenic power.

Personal Observations.

My own observations have extended over a series of 200 cases. They were undertaken at the Jenner Institute of Preventive Medicine, and I was much helped by the kind assistance and great experience of Dr. Allan Macfadyen, the Director of the Institute, and Dr. R. T. Hewlett, to both of whom I take this opportunity of tendering my most cordial thanks. I desire also to express my grateful thanks for the facilities offered me by the Institute.

The greatest care was taken to select cases for examination in which the conjunctiva was both subjectively and objectively normal, that is, smooth, of normal colour and consistence, with total absence of any smarting or pricking or morning gumming of the lids. Cases were further chosen with a view to difference in surroundings; thus, a certain proportion of cases were taken from people living in the country far away from any town, whilst others were taken from people living in London. As might be expected, there was a

[1] Randolph. *Arch. of Ophth.* Vol. xxvi. 3. 1897.
[2] *Ibid.*

larger proportion of sterile tubes amongst the country than amongst the town cases.

The following method was employed to carry out the examination :—A platinum looped needle, after careful sterilisation in the flame, was rubbed well over the conjunctival surface of the lower lid and over the bulbar conjunctiva. The irritation caused by the presence of the wire soon induced a slight flow of tears, and thus a washing of the contents of the sac was made to pass over the needle. Care was taken to prevent any contamination of the wire from the edges of the lids or eyelashes, the experiment being repeated if such an accident occurred. A second inoculation was also made in cases where one was not deemed sufficient. As regards the use of the platinum wire loop as a means of testing the conjunctival surface, the chief advantage lay in its easy sterilisation. By forming a fair-sized loop, a large amount of the wire surface was exposed, and if many organisms were present it would be impossible for the wire to remain uninfected. The most perfect method would undoubtedly have been to employ a camel's hair pencil; but it would have presented considerable difficulties in the way of efficient sterilisation, and it was considered that the results obtained by the wire loop would give a sufficiently accurate estimate of the bacterial condition of the sac.

Further, the platinum loop has been the method usually adopted in other researches of this kind.

Loeffler's blood serum was the medium selected as the one most suitable for the primary inoculations. This medium also possessed a special advantage on account of the frequent appearance of the xerosis bacillus, an interesting fact to which I shall refer again.

The following results were obtained: — Two hundred primary inoculations were made on tubes of Loeffler's blood serum. One hundred and fifty-nine tubes presented growths after inoculation. Forty-one tubes, or 20·5 per cent., remained sterile after inoculation.

The most interesting growth from a bacteriological point of view was that of the so-called *xerosis bacillus*, which was found present in no less than 118, or 74·2 per cent., out of

the 159 of the fertile tubes. In ninety cases, or 56·6 per cent. of the fertile tubes, this bacillus was found alone in pure culture, whilst in the remaining twenty-eight cases it was found mixed with other organisms. In each of these twenty-eight cases the xerosis bacillus was the predominating growth, and, with very few exceptions, the other micro-organisms were only present as one or two scattered colonies.

The following pyogenic cocci were isolated:—

The *st. pyogenes albus* was found in six cases, or in 3·7 per cent. of the fertile tubes, or 3 per cent. of the total 200 tubes. It was only found in pure culture twice. In the other four cases it occurred as one or two small isolated colonies in company with the xerosis bacillus.

The *st. pyogenes aureus* was found in one case. It occured in pure culture as a single small colony.

The *st. pyogenes citreus* was found in three cases, or 1·8 per cent. of the fertile tubes, or 1·5 per cent. of the 200 tubes. It was isolated in pure culture in one case, and then appeared as a fair sprinkling of small yellow colonies. In the remaining two cases it occurred as one or two colonies mixed with the xerosis bacillus.

The *st. cereus albus* appeared in four cases, or 2·5 per cent. of the fertile tubes, or 2 per cent. of the 200 tubes. In each of the four cases it grew as one or two small whitish colonies in company with the xerosis bacillus.

Fraenkel's pneumococcus was found in two cases. In both instances it appeared in pure culture.

Thus only sixteen tubes, or 8 per cent. of the 200 tubes, contained growths of the pyogenic cocci, and only in four tubes did they appear in pure culture. In every case, with the exception of one tube of st. pyogenes citreus, the number of colonies present were few and far between, being in some instances limited to a single colony. The pyogenic properties of the st. pyogenes citreus and the st. cereus albus, are comparatively slight, and, if we except these growths, we have only left seven tubes containing the st. pyogenes albus, and the st. pyogenes aureus, two of the most important varieties of the pyogenic bacteria.

In order to test the local virulence of the pyogenic organisms which were isolated, inoculations were made into the conjunctival sacs of guinea-pigs and rabbits, and in every case the result was negative and no reaction whatever occurred. The inoculations were made chiefly by injecting hypodermically into the conjunctival sac a powerful dose of the organism freshly grown in peptone broth. In a few cases another method was employed; viz., a platinum loop well smeared over with a 24-hours-old agar culture of the organism was well rubbed over the conjunctival surface. In one case of the st. pyogenes albus both methods were employed within four days of each other and fresh cultures were used in each case, but no reaction followed either inoculation. I regret to say that no inoculation was made with the st. pyogenes aureus, which was found in one case, but, having regard to the absence of results in experimenting with the other varieties of pyogenic cocci, I greatly doubt whether I should have met with any greater success with the aureus.

With reference to this point, it is interesting to note that Leber,[1] Weeks,[2] and others have inoculated the st. pyogenes aureus into healthy human conjunctival sacs without any result. No inoculations were made with the pneumococcus of Fraenkel. Both cases occurred in the same patient, and the growth died out in three days before I had an opportunity of testing its virulence.

So far as my investigations go, therefore, they point strongly to the fact that the pyogenic cocci only occur infrequently in the normal conjunctival sac, and that when found in this situation, they are either of a non-virulent type or, at any rate, speedily lose their pyogenic properties after their entry into the conjunctival sac.

The following variety of micro-organisms other than the pyogenic cocci and the above-mentioned xerosis bacillus were isolated:

The *st. epidermidis albus* was isolated in fourteen cases.

[1] *Die Bedeutung der Bakt., &c.* Seventh Intern. Ophth. Cong., Heidelberg, 1888.

[2] *Arch. of Ophthal.*, vol. xvi, p. 376.

In ten cases it appeared in pure culture, whilst in four cases it occurred as a few small colonies mixed with the xerosis bacillus. From six of these cases fresh sub-cultures were made and inoculated into the conjunctival sacs of guinea-pigs after the methods above enumerated. In each case the result was negative. It is generally conceded that this coccus is a harmless, inert variety of the st. pyogenes albus, and, consequently I have not reckoned it with the list of the pyogenic cocci.

In twenty-one other cases various other non-pathogenic bacteria, resembling the ordinary saprophytes of air and water, were found. Eleven cases occurred in pure culture, whilst the remaining ten cases appeared as one or two colonies mixed with the xerosis bacillus. There were four or five varieties of these bacteria, and an inoculation of each variety was made into the conjunctival sac of a guinea-pig, without, however, causing any reaction.

Sarcina alba and sarcina flava were found in six cases. In three cases sarcina was the only growth isolated, and in the other three cases it occurred with the xerosis bacillus.

Two varieties of torula were found in one case as the only growth present.

In eight cases various non-pathogenic bacilli were encountered. These included two cases of a large sporing bacillus, four cases of a large bacillus liquefying blood serum, and two cases of bacilli belonging to the mesentericus group, viz., the bac. mesentericus ruber and fuscus. One case of the bacillus which liquefied blood serum was inoculated into the conjunctival sac of a guinea-pig, but without any result.

The chief features of this report which I wish to bring under notice are :—

(1) The frequency with which the so-called xerosis bacillus was found.

(2) The comparative infrequency of pyogenic micro-organisms and the non-virulent type presented.

(3) The considerable number of sterile tubes.

1st. *With regard to the xerosis bacillus.*

Although the bacillus has been found by Uhthoff as an

occasional inhabitant of the normal conjunctival sac, yet other observers such as Franke and Eyre[1] have failed to isolate it. I believe that the frequency of its presence may have been overlooked because of the difficulty of growing the primary culture of the bacillus on any other medium than blood serum. For this medium the xerosis bacillus has the greatest partiality, and both primary and secondary cultures grow upon it with great freedom. Upon agar-agar it will seldom or never grow as the primary culture, and even secondary cultures form only a faint translucent film, whilst in gelatin the organism will never grow as a primary culture, and very rarely at all as a secondary culture.

The morphological resemblance of the xerosis bacillus to the Klebs-Loeffler bacillus makes it an easy growth to recognise, whilst the appearance of its colonies on blood-serum are quite characteristic. It is impossible in this paper to discuss the possible identity of the xerosis bacillus with the Klebs-Loeffler bacillus or the pseudo-diphtheritic bacillus, but the frequency with which I met this most interesting organism proves it to be the most common and universal resident of the normal conjunctival sac.

For a long time it has been gravely doubted whether this bacillus has any pathological connection with the disease known as xerosis. It is true that cases of this disease always show large numbers of this bacillus; but I have found many instances presenting just as free a primary growth of the bacillus in the healthy normal conjunctival sac as can be procured from cases of the disease itself. It is well known that the bacillus has no pathogenic properties in guinea-pigs and rabbits, and I have satisfied myself of the truth of this statement by inoculating fresh cultures into the conjunctival sacs of these animals.

The frequency with which this organism was encountered (118 cases, of 159 fertile tubes) proves conclusively that the bacillus can have nothing to do with xerosis. Little seems to be known of its presence in other parts of the body.

[1] *Journal of Path. and Bact.*, vol. iv., 1, 1896.

It has been found by Neisser in wounds and ulcers, but in no other situation has it been isolated in the numbers and frequency with which I have found it in the normal conjunctival sac. Its name, therefore, is most inappropriate, and I would suggest that some other more suitable term be found for it, such as the "*bacillus conjunctivæ communis.*"

Another point which the frequent appearance of this bacillus suggests is the possibility of mistaking it for the true Klebs-Loeffler bacillus in cases of membranous conjunctivitis when examination is being made for the latter. The morphological resemblance between the two when young and stained with methylene blue is very striking, and unless it is a skilled observer who undertakes the examination, or there is a good specimen of the true Klebs-Loeffler bacillus handy for the sake of comparison, the chances of error are very large, and, though I have no positive example, it seems possible that such an error is not infrequently made, and that cases of severe membranous conjunctivitis have been considered to be of diphtheritic origin, on account of the xerosis being mistaken for the Klebs-Loeffler bacillus. If the observer is not a practised person at the differential diagnosis, the only safe and certain method, and the quickest in the end, is to inoculate a recent pure culture of the suspected bacillus into some animal such as a guinea-pig. Death will result in a few hours if the case be one of true diphtheria.

Eyre, in his excellent monograph on the xerosis bacillus, states that the colonies of the primary culture do not appear visible for thirty-six to forty-eight hours after incubation. According to my experience this is somewhat too lengthy a period. In nine cases of pure xerosis the colonies were recognised and were very plainly visible twenty hours after the tube was placed in the incubator. In two other cases I noted the appearance of colonies twenty-two hours after incubation. Both were pure cultures of xerosis. In two or three of these eleven cases, in order to be quite certain of this point, specimens were stained, mounted, and recognised as undoubted xerosis under the microscope. On the other hand, I noted down in one case that no colonies were visible

to the naked eye eighteen hours after incubation, and in a certain number of cases I was certainly unable to detect signs of growth twenty-two hours after incubation. All my cases, however, exhibited well-marked colonies thirty hours after incubation; so that I should say that the usual time that xerosis makes its first visible appearance as a primary culture lies between twenty to thirty hours. All secondary cultures appear somewhat earlier than this, and if blood-serum or blood-smeared agar be employed abundant growth will be always apparent in less than twenty-four hours.

As regards Neisser's[1] method of staining the Klebs-Loeffler bacillus, I have always found it answer in an equally admirable manner with the xerosis bacillus. It has been claimed for this stain that it will serve as a point of differential diagnosis between the xerosis and Klebs-Loeffler bacillus, in that the former does not react to the stain unless of twenty-four hours' growth or more; whereas the Klebs-Loeffler bacillus will exhibit the granules from the time of its earliest appearance. This has not been my experience, for I succeeded in obtaining very good granular staining by this method in one case of xerosis of twenty-two hours' growth. This was a primary culture, and the specimen was therefore secured at the very earliest appearance of the growth.

Lastly, Eyre tells us that the xerosis bacillus, when grown in nutrient broth, produces slight alkalinity of this medium, and that this may serve as a useful point in the differential diagnosis between the xerosis bacillus and the Klebs-Loeffler bacillus, which invariably produces an acid reaction. I have in several cases grown xerosis on litmus agar, but have never succeeded in obtaining the slightest alteration in colour, whilst the Klebs-Loeffler cultures always produce marked reddening of the litmus. This difference in the behaviour of xerosis and Klebs-Loeffler bacilli, when grown on litmus agar, will serve as a practical diagnostic test and be more easily recognised than their reactions when grown in broth.

2nd and 3rd.—In briefly discussing the second and third

[1] *Zeitschr. f. Hyg.*, xxiv. 1897.

points, viz., *the non-virulence and comparative infrequency of pyogenic cocci and the large number of sterile tubes* (41 in 200), I shall endeavour at the same time to draw some definite conclusions with regard to the utility of antiseptics in ophthalmic surgery.

It will be noted that the streptococcus, which is probably the most potent and dangerous of all the pyogenic cocci, was entirely absent. Nothing like it was isolated in any of the 200 cases. This experience endorses that of other observers, and I think there can be no doubt that this micro-organism is *never* found in the healthy conjunctival sac, and that thereby one considerable element of danger in dealing with wounds involving destruction of the conjunctival epithelium is eliminated.

Now, in trying to form an estimate of the danger of wounds of surface where pyogenic cocci may be found, there are a good many factors to bear in mind beyond the mere presence of such micro-organisms.

Pyogenic organisms are not capable of themselves of causing suppuration. There must be, in addition, certain favourable conditions, both as regards the tissue in which the microbes lie and as regards the bacteria themselves.

These conditions have been set forth, in a most explicit manner, by Mr. Watson Cheyne in his work on "Suppuration and Septic Diseases."

As regards the tissue, we have to consider conditions which may prejudice the general vitality of the patient or the local vitality of the part. Upon the former point I need say no more in this paper, but with reference to the latter it is important to note that this local vitality, or power of offering resistance to the growth of pyogenic organisms, varies greatly in different tissues of the body. Thus, the endothelial cells of the peritoneum exhibit this vitality in a marked degree, and present a strong contrast to the more feeble resistance offered by the connective tissue cells of synovial membrane.

I shall point out later that the conjunctival epithelium seems to possess a great local vitality, similar to that of the peritoneal epithelium.

As regards conditions affecting the bacteria themselves, we have to consider the important factors of dose and virulence. Mr. Watson Cheyne has pointed out, and shown by direct experiment, that the severity of the affection resulting from the introduction of pyogenic bacteria varies directly, within certain limits, with the amount of virus introduced. These certain limits are constituted by the susceptibility of man to the influence of the virus and by the general and local vitality of the tissues. As regards virulence, there can be no doubt that the virulence of pyogenic micro-organisms diminishes with their age; so that we have most to fear from organisms that have been recently introduced.

Let us now consider the condition of the conjunctival sac with regard to these three factors:—(1) The local vitality of the conjunctival membrane; (2) the dose; and (3) the virulence of the contained organisms.

(1) *The local vitality of the conjunctival sac.*—The conjunctival sac is constantly exposed to contamination from without by particles of dust and all varieties of extraneous material. It is also in constant danger of infection from the nose by means of the lacrymal passage. Pyogenic organisms which have thus obtained an entrance are carefully fostered in its recesses by its peculiar anatomical conformation. For these reasons the conjunctival sac has always, until recently, been regarded as a species of anatomical sink—a nidus for any passing microbe. The reverse is the actual case. Instead of teeming with all manner and conditions of micro-organisms, the conjunctival sac is singularly devoid, at any rate, of living bacteria. Forty-one of two hundred tubes were found absolutely sterile, and many more only presented a few discrete colonies. The conjunctival sac may possibly never be quite sterile, although, for my own part, I believe that this is frequently the case; but at least the sterility of these forty-one tubes is strong evidence that living micro-organisms were few and far between. Indeed, one of the most striking features of my own observations was the small number of colonies in the large majority of the tubes; the only micro-

organism that was often plentiful being the so-called non-pathogenic xerosis bacillus. It is to be further remarked that other observers, such as Leber, Fick, and others, have failed to obtain growths of any description in a certain number of inoculations from the healthy conjunctival sac. In the face of these facts one cannot but believe that the conjuctival epithelium exerts a remarkable and detrimental influence upon the life and growth of bacteria.

(2) and (3) *Dose and Virulence.* If we pass on to the consideration of dose and virulence we obtain still further evidence of the great local vitality of the conjunctival membrane. In spite of its constant exposure to infection, pyogenic organisms of any description, including the st. epidermidis albus, were only found in thirty tubes. In almost every case the number of colonies was very limited. It is possible, and I do not wish to dispute it, that pyogenic organisms would have been found in all the 200 cases could the bacterial examination have been made absolutely complete, but the fact that none were found in 170 cases, and, if we exclude the st. epidermidis albus, in 184 cases, points to the conclusion that, if present, they were very few in number. Consequently we may conclude that in the healthy conjunctival sac we have only to deal with a very small number of pyogenic bacteria. Further, the negative results of inoculation of fresh cultures of these bacteria into the conjunctival sacs of guinea-pigs and rabbits showed that these bacteria had, in a great measure at least, lost their virulence.

The sum of these facts seems in short to warrant the conclusion that the healthy conjunctival sac, probably chiefly by its epithelium, but also possibly in part by means of the lacrymal secretion, is remarkably resistant to the growth and life of pyogenic bacteria, and, as a further deduction, that wounds of the healthy conjunctiva are not nearly so liable to suppurative processes as similar wounds in many other parts of the body.

Finally, let us briefly consider the bearing of these results upon the question of antiseptics in ophthalmic surgery.

In the first place, I doubt if any other branch of surgery can show such brilliant results as regards the general absence of suppuration as ophthalmic surgery. Suppuration in an eye after a well-performed operation with a normal conjunctiva is a very rare occurrence. It is not indeed the frequency of suppuration, but the hopelessly disastrous results to an eye if suppuration occurs, that makes the question of its exclusion of paramount importance, and has raised the question of the utility of antiseptics to one of the highest moment. In many other branches of surgery moderate suppuration prejudices and delays healing, but may not ultimately affect the successful result. With the eye it is different, and the onset of suppuration is the most dreaded misfortune that can befall the ophthalmic surgeon.

Secondly, as a result of much discussion and numerous papers, it has been generally conceded that antiseptics of such strength as to act as thoroughly effective germicidal agents are too irritating and, by their action in lowering the local vitality of the part, productive of more harm than good. Thus it has come about that a large number of the most experienced ophthalmologists have entirely discarded the use of such agents as mercury in their practice, preferring the risk, if any, that may be entailed by their avoidance to the prejudicial effects observed as their consequence.

The abolition, therefore, of antiseptics in modern ophthalmic surgery has its *raison d'être*, not so much from a disbelief in the efficacy of antiseptic solutions nor in a disregard of the principles which promote their use, but in a justifiable dread that any advantages they may possess are counterbalanced by still greater disadvantages.

The bacterial condition of the healthy conjunctival sac, as I have already detailed it, gives us, I venture to think, a further and still more reasonable ground for the abolition of antiseptic solutions in the treatment of eye wounds. We have, in short, in the conjunctiva, a membrane that is working on the surgeon's behalf, that possesses inherent antiseptic qualities, and nothing but harm can result from the use of any chemical agent which depresses and lowers its vitality,

and thereby renders it less resistant to the growth of pyogenic organisms. The very fact of the exemption from suppurative processes that eye surgery has always enjoyed is in itself a strong proof of the antiseptic nature of the conjunctiva. Without doubt, the number of cases of suppuration has been still further reduced with the recognition of the importance of asepsis, but before the days of asepsis eye surgery always enjoyed great immunity in this respect. This is the more remarkable when we consider that if an eye is bandaged after operation, as is commonly done, any bacteria that may happen to be in the conjunctival sac are brought into close and constant contact with the wound service, whilst there still remains the open channel of infection from the nasal fossa by means of the lacrymal passage.

Thus, I would base and urge the abolition of antiseptics in ophthalmic surgery on the ground that the strongest antiseptic is the healthy conjunctiva itself, and in order to eliminate suppuration as far as possible from the list of accidents that may occur after operations we have only two factors to consider: (1) Perfect asepsis on the part of the operator, his instruments, dressings, &c., and (2) the healthy condition of the conjunctival sac.

Enough I hope has been said to show that we may part with antiseptics in ophthalmic surgery, not with an uneasy feeling that we had better be without them on account of the local disturbance that they produce, but with the satisfactory conviction that we do not need them on account of the antiseptic qualities of the conjunctiva itself.

I should add that the substance of this paper was read at the Edinburgh Meeting of the British Medical Association in 1898.

PRELIMINARY OBSERVATIONS ON THE OCCURRENCE OF THE BACILLUS ENTERITIDIS SPOROGENES (KLEIN) IN ULCERATIVE COLITIS AND IN THE NORMAL DEJECTA.

BY RICHARD T. HEWLETT, M.D.

In a "Report on an Inquiry into the Relations of Asiatic Cholera and Cholera Nostras,"[1] Klein isolated and described an anaerobic bacillus which he named the *bacillus enteritidis sporogenes*.

This organism was isolated from the evacuations of, and from milk consumed by, patients suffering from an epidemic diarrhœa which occurred in St. Bartholomew's Hospital, and which seemed to be traceable to the milk supply.

Further observations were made by Andrewes[2] on twenty cases of diarrhœa admitted into St. Bartholomew's Hospital, from the majority of which the *bacillus enteritidis sporogenes* was isolated.

Neither Dr. Klein nor Dr. Andrewes commits himself to a definite statement as to the relation of the organism to the disease, though the former speaks of "this diarrhœal organism," and the latter says, "Whether the bacillus in question is actually a cause of acute diarrhœa is not so far positively established, but there seems to be a distinct probability that such is the case."

Klein, in a more recent paper,[3] deals very fully with the morphology, biology, and distribution of this bacillus.

[1] Report of the Medical Officer, Local Government Board, for 1895-96, p. 197.

[2] Report of the Medical Officer, Local Government Board, for 1896-97, p. 225.

[3] Report of the Medical Officer, Local Government Board, for 1897-98, p. 210.

With regard to the last-named point, it was isolated from several cases of infantile diarrhœa and cholera nostras, and was found to be present in water, sewage, manure, and in eight out of ten samples of milk examined. In other cases of infantile diarrhœa, from which the organism was not isolated, Klein says: "Bacilli, as chains and as cylindrical rods, could be demonstrated in microscopic specimens of the bowel contents, that might have been *bacillus enteritidis* in a non-sporing state."

Lastly, he states, "I have since ascertained that in the intestinal evacuations of healthy individuals, tested by the same methods for the presence of the spores of this microbe, no positive result is obtained."

The *bacillus enteritidis sporogenes*, as described by Klein and Andrewes, occurs as a shorter or longer rod measuring between 1·6 and 4·8 μ in length, and about 0·8 μ in diameter, also linked together to form chains. It is strictly anaerobic, stains well by Gram's method, is motile and possesses several flagella, and under certain conditions spores freely. One of the most typical culture appearances is the change produced by it in milk. Inoculated into recent milk tubes and incubated at 37° C. anaerobically, in from twenty-four to forty-eight hours the surface of the medium is covered with tough, stringy, pinkish-white masses of casein, with entangled bubbles of gas, while the main portion of the tube is occupied by a thin, watery, colourless, clear or slightly turbid whey, with a few small lumps of casein at the sides and bottom of the tubes.

Gelatine and serum are both liquefied (the former only when spores are present), and spore formation occurs in these media but not in milk or agar.

In agar the colonies are "delicate semi-transparent and grayish, with smooth or wavy edges, which later show fern-like and dentate outgrowths" (Andrewes), or "are circular, flat, moist, grey and translucent, with a thicker and less transparent middle part, and a thinned-out and more transparent marginal portion" (Klein).

Gas is produced abundantly in both glucose agar and glucose gelatine. Inoculated subcutaneously in the abdomen

of a guinea-pig in doses of 1 c.c. of the whey per 200 to 300 grains of body weight it produces death in eighteen to twenty-four hours, and *post mortem* it is found that the hairs come away from the skin, which is green and gangrenous, that the subcutaneous tissues are green and gangrenous, or more or less dissolved, so that the skin is severed from the subjacent tissues, the cavity so formed containing gas, and more or less exudation, sometimes scanty, at other times thin, abundant, and sanguineous, in either case containing large numbers of bacilli mostly isolated but sometimes forming chains.

If the *post mortem* is delayed for six to twenty-four hours after the death of the animal, spore formation will be found to have taken place.

In order to isolate the organism, Klein makes use of milk cultures. Recent (not more than four days old) milk tubes are inoculated with a trace of the bowel contents, &c., heated in a water-bath for ten to fifteen minutes at 80° C., and then incubated anaerobically at 37° C.

If the *bacillus enteritidis sporogenes* be present, the milk tubes show the typical changes described above within forty-eight hours. The only other organism, according to Klein, which produces the same changes is the *bacillus butyricus* of Botkin, which, however, is non-pathogenic.

Another organism isolated by Klein,[1] and named by him the *bacillus cadaveris sporogenes*, has to be distinguished from the *bacillus enteritidis sporogenes*. The two organisms are morphologically very similar, and both stain by Gram's method, but the *bacillus cadaveris sporogenes* does not produce the typical changes in milk. In a culture in two to three days the milk below the cream layer commences to clear, and later this change proceeds rapidly, so that at the end of a week three layers are apparent, an upper of unchanged cream, a middle yellowish and watery, and a lower of precipitated casein. Its colonies on agar are also different, sending out ramifying, anastomosing threads from their margins, and it spores freely on agar in two to three days.

[1] *Centr. f. Bak.*, xxv., No. 8-9, p. 278.

Lastly, Herdman and Boyce state:[1] "We have shown also the frequent occurrence, in various shell-fish from the shops, of anaerobic spore-bearing bacilli giving characteristics of the *bacillus enteritidis sporogenes* recently described by Klein."

Twelve specimens of dejecta, &c. (kindly sent by Dr. Richard Legge, Derby County Asylum) from cases of ulcerative colitis, have been examined, and in each case the *bacillus enteritidis sporogenes* has been found to be present. Dejecta from a case of ordinary diarrhœa (also sent by Dr. Legge) and from a case of chronic dysentery were examined, and the *bacillus enteritidis sporogenes* was isolated from each. The dejecta from a case of irritant poisoning did not yield this organism.

These results being of some interest, it was thought advisable to examine some specimens of normal dejecta.

Four times I have examined my own dejecta, and on two occasions have isolated the *bacillus enteritidis sporogenes*. I have also examined four specimens from healthy individuals (forwarded by Dr. Legge[2]) and five specimens from normal children (surgical patients in Great Ormond Street Children's Hospital with normal digestive tracts, kindly sent by Dr. Ralph Williams), and from all of these the *bacillus enteritidis sporogenes* has been isolated.

Throughout the investigation the method of isolation adopted was that used by Klein, viz., recent milk tubes were inoculated, heated to 80° C. for ten to fifteen minutes, and then incubated anaerobically at 37° C. in well-stoppered bottles, the oxygen in which was absorbed by means of alkaline-pyrogallol (Buchner's method). All tubes were discarded unless they showed the typical changes (surface clot of shreddy casein with gas bubbles and clear whey)

[1] "Observations on the Normal and Pathological Histology and Bacteriology of the Oyster." Royal Society, January 19, 1899.

[2] Dr. Legge informs me that he has examined a number of specimens of dejecta from healthy individuals by means of anaerobic milk cultures and microscopical preparations therefrom, and as far as he could judge from these, the *bacillus enteritidis sporogenes* seemed to be generally present.

within forty-eight hours; but, as will be seen from the protocols, nearly all the tubes inoculated curdled typically. From tubes showing typical changes guinea-pigs were inoculated with 2 to 4 c.c. of the whey and the pathogenic characters noted. In several instances sub-cultures were made from the milk tubes and from the guinea-pigs, with subsequent animal experiments. (The details of many of the experiments are given in the protocols at the end of this paper.)

In all cases the cultures were controlled by microscopical examination and staining by Gram's method. The typically curdled milks always showed a large non-sporing bacillus which stained well by Gram's method. Spores were never found in agar cultures, whereas they were present in serum cultures. The only differences I could detect between the organism isolated by myself and the *bacillus enteriditis sporogenes* as described by Klein, were that the former produced but little liquefaction (slight pitting only) of serum in a week, and that its virulence seemed slightly less, 1·5 c.c. of the whey per 200 grammes of body weight being required to produce death in a guinea-pig in twenty-four hours. As regards liquefaction, Andrewes' results and Klein's experiments show that this is a very variable phenomenon, while virulence depends not only on the strain of an organism, but also on the dose, and it was noticed that the number of bacilli present in the milk cultures varied very much, so that injecting the same volume of whey from different cultures, the number of organisms introduced, *i.e.*, the dose, was by no means identical. It is quite conceivable that in diarrhœa, &c., the number of organisms present and their virulence might be greater than under normal circumstances without the diseased condition being necessarily due to the activity of the bacilli. Another point noticed was the different appearances presented by a diffuse growth of the *bacillus enteritidis sporogenes* and widely separated isolated colonies on anaerobic agar surface cultures three to four days old. When the growth is quite confluent and diffuse it is semi-transparent and delicate, almost as delicate as a good growth of *streptococcus pyogenes*,

and grayish-white. When less diffuse, so that the colonies are just discrete, the growth is denser; when the colonies are further apart, Andrewes' description is exactly reproduced, while when there is a considerable interval between the colonies these are much denser and more cream-coloured, showing a granular central portion bordered by a narrow semi-transparent zone.

Klein's statement that in cases of infantile diarrhœa, from which the *bacillus enteritidis sporogenes* was not isolated, "bacilli, as chains and as cylindrical rods, could be demonstrated in microscopic specimens of the bowel contents, that might have been *bacillus enteritidis*, &c.," is doubtless true, but is, I think, applicable to any microscopic specimen of fæces; such, at all events, is my experience.

To sum up:—I have isolated the *bacillus enteritidis sporogenes* from the dejecta in twelve cases of ulcerative colitis, in one case of ordinary diarrhœa, one case of chronic dysentery, and in eleven out of thirteen specimens of normal dejecta from ten healthy individuals, and the conclusion I have formed is that this organism is probably an inhabitant of the normal digestive tract, and frequently to be found in the dejecta; considering the wide distribution of the organism and the resistant nature of its spores, this is not unlikely to be the case.

I have isolated the *bacillus enteritidis sporogenes* from road dust, from laboratory dust, from water, and from eight samples of milk out of fifteen examined, and the conclusion I have formed is that this organism is probably ubiquitous.

I cannot help thinking that the names chosen by Dr. Klein, *bacillus enteritidis sporogenes* and *bacillus cadaveris sporogenes*, are unfortunate. In the first place, by the rules of nomenclature adopted in natural history, trinomial names should as far as possible be avoided. Secondly, organisms have been already described under the names *bacillus enteritidis* and *bacillus cadaveris*, and this is likely to lead to confusion. Thirdly, by adopting the trinomial name, *bacillus enteritidis sporogenes*, some resemblance would be anticipated between it and the *bacillus enteritidis*, which is not the case.

Protocols of Experiments.[1]

I.—*Cases of Ulcerative Colitis.*

(1) *From Derby, J.*

March 14—Five milk tubes inoculated.

March 15—All inoculated tubes curdled typically. Control tubes unaltered. Two guinea-pigs inoculated with 1 c.c. and 3 c.c. of the whey respectively.

March 17—Guinea-pig inoculated with 3 c.c. dead, with typical *post-mortem* appearances.

(2) *From Derby, R.*

March 15—Three milk tubes inoculated.

March 16—All the milk tubes curdled typically. A guinea-pig inoculated with 1·5 c.c. A swelling and ulceration developed at the seat of inoculation, but the animal did not die.

(3) *From Derby, K.*

March 24—Three milk tubes inoculated. (Sunday intervened.)

March 27—All the milk tubes curdled typically. Surface-agar cultures (anaerobic) made and 2 c.c. of the whey inoculated into a guinea-pig.

March 29—Guinea-pig: induration at seat of inoculation. Agar cultures show grayish, semi-transparent colonies composed of a large bacillus staining by Gram's method. A milk tube inoculated from an isolated colony.

March 30—Guinea-pig: large swelling at seat of inoculation. This guinea-pig died April 6. Milk tube from agar culture (March 29) curdled typically.

II.—*Case of Diarrhœa.*

(1) *Case of ordinary diarrhœa from Derby.*

March 17—Four milk tubes inoculated.

March 18—All the inoculated tubes are curdled typically; two control tubes remain unchanged. (Being Saturday, inoculation postponed until Monday.)

[1] In all cases recent, *i.e.*, not more than five days old, milk tubes were inoculated, heated in a water-bath to 80° for ten to fifteen minutes, and then incubated at 37° C. anaerobically by means of alkaline pyrogallol. Microscopical specimens were made and stained both with weak carbol-fuchsin and by Gram's method from the milk-tubes and animals, and in all cases showed the presence of a large bacillus, resembling the *enteritidis sporogenes* in morphology and in staining by Gram. With a few rare exceptions the cultures were pure.

In all cases, unless the contrary is stated, the cultures were incubated anaerobically.

March 20—A guinea-pig (600 grm.) inoculated with 4 c.c. of the whey.

March 22—Guinea-pig died to-day with typical *port-mortem* changes of enteritidis. Cultures made on surface agar (anaerobic).

March 27—Agar surface cultures show grayish-white semi-transparent colonies with somewhat crenated margins. The colonies are composed of a large bacillus staining by Gram. No spores present. Re-inoculated into milk tubes.

March 28—Milk tubes curdled typically. A guinea-pig inoculated with 3 c.c.

March 29—Guinea-pig dead with typical *post-mortem* appearances.

III.—*Normal Dejecta.*

(1) *From Derby.*

March 14—Six milk tubes inoculated, heated, and incubated at 37° C. with control (uninoculated) tubes.

March 15—All the inoculated tubes are curdled typically, the control tubes being unchanged.

Two guinea-pigs inoculated with 2·5 c.c. and 1 c.c. of the whey respectively.

March 16—Guinea-pig inoculated with 2·5 c.c. dead. *Post-mortem* changes typical of *enteritidis*. Large bacillus staining by Gram present in exudation.

March 19—Guinea-pig inoculated with 1 c.c. dead.

(2) *From Derby.*

March 14—Six milk tubes inoculated, &c., as in (1).

March 15—Three of these milk tubes curdled typically. A guinea-pig inoculated with 2·5 c.c. of the whey.

March 16—Guinea-pig dead with typical *post-mortem* appearances and large bacillus present in exudation.

(3) *Self.*

March 16—Four milk tubes inoculated, &c., as in (1).

March 17—All four tubes curdled typically. Two guinea-pigs inoculated with 1 c.c. and 3 c.c. of the whey respectively.

March 18—Guinea-pig inoculated with 3 c.c. dead, with typical *post-mortem* appearances.

(4) *Self.*

March 28—Three milk tubes inoculated, &c., as in (1).

March 29—Two out of the three tubes curdled typically.

Two guinea-pigs inoculated each with 2·5 c.c. Anaerobic cultures on agar also made, but no growth occurred.

March 30—One guinea-pig dead with typical *post-mortem* appearances; agar surface cultures (anaerobic) made from exudation.

The second guinea-pig has a large "gas-bag" in abdomen, and died April 10.

April 4—Agar surface tubes inoculated from guinea-pig on March 30 show round cream-coloured colonies, the margins of which are thinner than the centre. The colonies are composed of large bacilli, which stain well by Gram's method. Milk tubes were inoculated from these colonies.

Surface agar (aerobic) cultures were also made, but no growth ensued, so that the organism is strictly anaerobic.

April 6—Milk tubes inoculated from agars on April 4 are curdled typically. Sub-cultures made on surface agar (anaerobic) and a guinea-pig inoculated with 2 c.c. of the whey.

April 7—Guinea-pig dead with typical *post-mortem* appearances. Surface agar (anaerobic) cultures made.

April 10—Cultures on agar from milk (April 6) show round cream-coloured colonies with thicker central granular portion and narrow thin border; no out-growths. Growth consists of a large bacillus staining by Gram; no spores. One milk tube inoculated (*a*). Agar tubes inoculated from guinea-pig on April 7 show similar colonies. One milk tube inoculated (*b*).

April 12—Milk tubes *a* and *b* (April 10) curdled typically. Both show a large non-sporing bacillus; no other organisms. Surface agar cultures, aerobic and anaerobic, made from each, and two small guinea-pigs inoculated respectively with 1·5 c.c. from each tube.

(5) *Child* (*F., aged* 1½. *Cleft palate. Gt. Ormond St.*).

March 22—Four milk tubes inoculated, &c., as in (1).

March 23—All tubes curdled typically. Controls remain unaltered.

Stab sugar agar and surface-agar (anaerobic) cultures made.

Guinea-pig (510 grm.) inoculated with 3 c.c. of the whey.

March 24—Guinea-pig dead with typical *post-mortem* appearances. Cultures made on surface agar (anaerobic).

March 27—Surface agar cultures of March 23 and March 24 show grayish-white, semi-transparent colonies with somewhat wavy margins. Cultures are apparently pure, and the growths consist of large bacilli which stain well by Gram. No spores found in condensation water.

Sub-cultures in milk made from isolated colonies.

March 29—Milk tubes curdled typically. Guinea-pig inoculated with 2·5 c.c. of the whey.

March 30—Guinea-pig dead with typical *post-mortem* appearances.

(6) *Child* (*M., aged* 7 *months. Talipes. Gt. Ormond St.*).

Details and results of experiments same as No. 5 up to and including March 27.

March 28—Milk tube curdled typically. Guinea-pig inoculated with 3 c.c. of the whey.

March 29—Guinea-pig dead with typical *post-mortem* appearances. Agar surface cultures (anaerobic) made.

April 4—Agar tubes of March 29 show typical colonies. Milk tubes inoculated from isolated colonies, also *aerobic* agar surface tubes.

April 6—Milk tubes curdled typically. 2 c.c. of whey inoculated into a guinea-pig, and sub-cultures on surface agar (anaerobic) made. Aerobic cultures show no growth.

April 7—Guinea-pig dead ; typical *post-mortem* appearances.

April 10—Surface agar cultures of April 6 show typical colonies of *b. enteritidis sporogenes.*

(7) *Child* (*M., aged* 3. *Contraction after burn. Gt. Ormond St.*).

Details and results of experiments same as No. 6 up to and including March 28.

March 29—Guinea-pig has gas-bag in abdomen.

March 30—*Ib.*

(8) *Child* (*F., aged* 11. *Amputation for tubercular knee. Gt. Ormond St.*).

Details and results of experiments same as No. 5 up to and including March 27.

March 29—Milk tubes inoculated from agars on March 27 show no change, and therefore discarded. Fresh milk tubes inoculated from the same agars; these also showed no change.

(9) *Child* (*M., aged* 6. *Old tubercular knee. Gt. Ormond St.*).

Details and results of experiments same as No. 5 up to and including March 27.

March 29. Milk tubes inoculated from agars on March 27 show no change, and therefore discarded. Fresh milk tubes inoculated from the same agars.

March 31—Milk tubes curdled typically. The Easter holi-

days intervening, the tubes were kept in the incubator under anaerobic conditions until April 4, when guinea-pig was inoculated and surface agar cultures (anaerobic) made.

April 6—Guinea-pig dead with typical *post-mortem* appearances. Agar surface cultures (anaerobic) made from guinea-pig. Agar cultures of April 4 show *enteritidis* colonies.

April 10—Agar tubes of April 6 show *enteritidis* colonies. No spores in condensation water.

PHOTOGENIC BACTERIA.

BY J. E. BARNARD.

THE investigation here described was undertaken with the object of determining the conditions under which certain bacteria have the power of producing and emitting light. The morphology and characteristics of these organisms have been carefully observed, but the present paper must be regarded as a preliminary one, owing to the inherent difficulty of certain phases of the research. The main object in view has been rather to determine fas ar as possible the cause of the phosphorescence and the conditions necessary for its production than to identify fresh species, and it is hoped in a future paper to still more fully describe some physical and chemical experiments at present in progress which bear on this branch of the subject. It might be mentioned that all the results here given have been determined by independent observation without regard to the results of others.

It is, perhaps, in such simple organisms as bacteria that the phenomenon of light production is most remarkable. In higher animals with the same power there are special organs involved which emit light as the result of some complex reaction at present but imperfectly understood, but in bacteria no such special organs can be said to exist, and their unicellular structure points rather to their power of phosphorescing, by which term it is sometimes alluded to, being an accompaniment of their functional activity, much as heat is in our own bodies.

The subject of phosphorescence, in whatever department of nature it occurs, is one of great interest. The phenomenon itself is so striking that whether considered from a scientific or

a popular point of view it attracts attention, and has at various times given rise to a great amount of speculation, Pliny even having known and noted the appearance of pholas, the rock-boring mollusc. The most notable reference to the subject is by Darwin. It is suggestive of the trouble even he must have experienced in accounting for its production, as it is referred to in his chapter on "Special Difficulties of the Theory of Natural Selection." This chapter opens by saying: "Although we must be extremely cautious in concluding that any organ could not have been produced by successive, small, transitional gradations, yet undoubtedly serious cases of difficulty occur." The electric organs of fishes are then considered, and many of the statements in reference to that subject are of interest when considered in relation to phosphorescence. "The luminous organs which occur in a few insects, belonging to widely different families, and which are situated in different parts of the body, offer, under our present state of ignorance, a difficulty almost exactly parallel with that of the electric organs."

Phosphorescence occurs in many living creatures, but the largest number are marine. The well-known emission of light by fire-flies and the glow-worm are notable exceptions, however, and have formed the basis of a great many scientific investigations.

It has also been observed in some phanerogamic plants, such as the nasturtium, marigold and lily, but the instances on record are of doubtful authenticity, and even if they occur are probably due to some special conditions, and can hardly be regarded as a function of the plant itself. It is worthy of note, however, that nearly all plants which have been regarded as phosphorescent have red or yellow flowers. In the cryptogamic plants the examples are more numerous and would appear to be more intimately associated with their life history. The phosphorescence that sometimes appears on the stumps of old trees would appear to be due to certain species of moulds.

The phosphorescence of chemicals and minerals is well known. Barium, strontium and calcium sulphides, for instance, are the best known examples, the barium salt

having been named "Balmain's luminous paint." But in these there is some evidence of chemical changes as their absorbing and re-emitting power gradually decreases. Friction, heat, light and electricity will cause certain bodies to become phosphorescent, and cooling the body may prevent the continuance of the luminosity. By cooling the centre of a plate which has been coated with sulphide of calcium, light will make it phosphoresce everywhere except in the place where it has been cooled. Heat increases the luminosity at first, but it afterwards dies out more quickly than where the plate has not been heated.

Geissler first discovered that vacuum tubes emit light for some time after the discharge through them has ceased, and the most recently observed examples of phosphorescence are to be obtained by the action of kathode and similar radiations on various substances. The emission of light by photographic plates during the process of development is worthy of note as it is more nearly allied to bacterial phosphorescence, the gelatine of the plate apparently absorbing oxygen while under the influence of an alkaline pyrogallic acid solution. Even this, however, may be an emission of light which had been previously absorbed, and not the result of chemical activity between the substances involved.

All those bodies or organisms which emit light that cannot be attributed to combustion, using the word in its usually accepted sense, are referred to as phosphorescent. It is, however, very difficult to demarcate the boundary that divides phosphorescent bodies from those in which rapid absorption of oxygen and evident chemical change are taking place. Phosphorous itself, for instance, is a case in point, as owing to it being able to combine with oxygen at a relatively low temperature, the light it emits is sometimes associated with phosphorescence. In this case, however, a rise of temperature does not take place, and the chemical activity is accompanied by increased radiant heat. In phosphorescent organisms there are no radiant heat waves produced, and this fact constitutes their chief characteristic. We have light without heat.

The phosphorescence of marine animals is well known,

and the examples to be found in sea-water are numerous. The best known in northern latitudes is the one belonging to the lowest sub-kingdom of animals, noctiluca miliaris. This organism appears to be the chief cause of the diffused luminosity of the sea, these animalcules existing in immense myriads in the waters of the ocean. They can be obtained in large numbers in an ordinary tow net, and when transferred to a jar of sea-water they soon rise to the surface, forming a thick scum. The slightest agitation of the jar causes them to emit light in flashes of short duration, and they require short rest before they can again exhibit their power. Chemical or electrical stimulation also has a similar effect. Professor Allman[1] was able to observe the phosphorescence of these organisms under the microscope with a half-inch objective, by adding a drop of alcohol to the water containing them; and was able to determine the special seat of the phosphorescence. Another well-known light-producing animal is the rock-boring mollusc, pholas. In some marine animals, the light does not appear to be confined to one place but is distributed over their bodies. It is most likely that in such cases the light is not emitted by the animal itself, but is due to a parasitic growth of photogenic bacteria.

The term "phosphorescence," as applied to bacteria, is perhaps somewhat misleading, as it is likely to be regarded as analogous to the phenomena recently so prominently brought forward in connection with electric discharges in high vacua. As pointed out by Mr. Herbert Jackson in his discourse on "Phosphorescence" before the British Association (1898 Meeting), phosphorescing bodies may be divided into two classes. In one there is chemical change, such as oxidation, and in the other no change is apparent. Photogenic bacteria belong to the first-mentioned class, and, as will be seen later, free oxygen is absolutely necessary for their growth.

It is perhaps unfortunate that the term "phosphorescent" has been applied to these organisms at all. Had they been generally referred to as "photogenic" their

[1] Allman. *Quarterly Journal of Microscropical Science*, vol. xii., 1872, p. 327.

particular power would have been more accurately indicated. However, the former term seems to have been extensively used in connection with those bodies which have the power of emitting light without any appreciable heat other than that due to the heating effect of any light waves, and in this sense bacteria are undoubtedly correctly described when spoken of as "phosphorescent." Roscoe[1] refers to the subject when discussing fluorescence and phosphorescence and says: "the fluorescence of bodies is closely allied to other phenomena to which the term phosphorescence is usually applied. These only seem to be cases in which the fluorescence does not disappear on the removal of the light causing it, but subsists for some sensible length of time."

It is necessary to point out, however, that there is a vital difference between the light-emitting power of bacteria and other marine organisms, and that of inert chemicals and minerals. Bacteria emit light, which is produced by themselves without regard to any extraneous source, but in the case of inert substances these have first to be exposed to a source of light, and then have the power of re-emitting light which is proportional in intensity and duration to the original source. In an inert phosphorescent body the molecules have the power of setting up light waves in the ether *after* a light has ceased to shine upon them from without, but in bacteria they themselves are the source of light. The power of almost any inert matter to set up light waves has lately been demonstrated in a most striking manner by Professor Dewar, who produced phosphorescence in a variety of substances after they had been cooled to the temperature of liquid oxygen and exposed to the rays of the electric arc.

The appearance of the light emitted by the relatively large marine animals, which have this power, is very like a high tension electrical discharge, such as from an induction coil. In the case of noctiluca, for instance, which is probably the most widely distributed of all light-producing organisms, the light is intermittent, only appearing in flashes after or

[1] Roscoe, "Spectrum Analysis."

during some disturbance of the water or irritation of the organism. It is possible that bacteria also do not continually glow as might be concluded from the appearance of an artificial culture. The fact that in sea-water, or in tube cultivation, they occur in enormous numbers renders the settlement of this point a most difficult one. The individual bacterium does not emit sufficient light to enable the observer to study it under the microscope by its own light, but in some cultures, and more particularly in young colonies in plate cultivations, a periodic increase and decrease in the light can be observed. It is therefore possible that the light is emitted at short intervals, but owing to the numbers present this does not appear probable to the casual observer.

The natural habitat of light-producing bacteria seems to be almost exclusively sea-water. In some few cases they have been observed as parasites on higher marine animals, such as crabs. Giard[1] found a pathogenic and phosphorescent bacillus on species of the genus talitrus. It lives in the abdominal cavity and enters the organs, ultimately causing death. During the disease the animal emits green light, which ceases a few hours after death. It is possible that some few infusoria and polyps do emit light which could be attributed to bacteria, as instances are not uncommon in which the light does not cease on the death of the animal, but it is in sea-water and on the bodies of dead fish that they are found in profusion. The most widely known source is the body of a dead herring or mackerel, but in this case the organisms are probably conveyed from the sea-water, as if the fish is well washed in fresh water directly it is caught no phosphoresence appears.

The organisms are apparently distributed throughout the water, that is they do not only appear at the surface, as samples of sea-water obtained at depths still contain them. In the tropics they sometimes occur in enormous numbers, even causing the sea to be phosphorescent over large areas, but in northern latitudes they occur in small numbers, and the phosphorescence of the sea can rarely, if ever, be attributed to them.

[1] Giard, Paris, 1889.

The bacteria at present recognised as photogenic are the following:—

Photobacterium phosphorescens (Fischer).
,, annulare (Fischer).
,, balticum (Fischer).
,, caraibicum (Fischer).
,, indicum (Beyerinck).
,, coronatum (Fischer).
,, degenerans (Fischer).
,, fischeri (Beyerinck).
,, glutinosum (Fischer).
,, javanense (Eijkmann).
,, luminosum (Beyerinck).
,, pflugeri (Beyerinck).
,, tuberosum (Fischer).
,, hirsutum (Fischer).
Bacillus cyaneo-phosphorescens (Katz).
,, smaragdeo-phosphorescens (Katz).
,, argenteo-phosphorescens I. (Katz).
,, ,, ,, II. (Katz).
,, ,, ,, III. (Katz).
,, argenteo-phosphorescens liquefaciens (Katz).
Bacterium pholas (Dubois).
,, pelagia (Dubois).
,, giardi (Billet).
Photobacterium delgadense (Fischer).
Photobacterium papillare (Fischer).

Phosphorescent vibrios have also been described as occurring in the river Elbe.

There is a good reason to believe that a certain number of the above organisms do not represent sharply differentiated species, but that they are in some instances identical, or at any rate very closely related. The nomenclature goes further than is justified by the hitherto ascertained facts, and we do not consider that the above classification will eventually hold good. The forms that are probably identical are referred to later on in this paper.

It is proposed in this paper to describe my observations upon the morphological and other characteristics of a number of these organisms, although the general remarks as to culture media, &c., may be taken to apply to all of them. Before going on to describe the characteristics of each organism however, some description of the culture media employed will be given, but any medium which

favours the growth of a particular organism only will be mentioned when referring to that organism.

Artificial culture media.—All known phosphorescent bacteria grow on ordinary peptone beef broth gelatine, but they do not all phosphoresce on that medium, and none of them give out their maximum amount of light. It was recognised that in sea-water itself would in all probability be found the necessary and best materials for their development. The chemical composition of sea-water was taken as follows :—1000 cubic centimetres contain :

Sodic chloride	26·439 grains
Potassic ,, ...	0·746 ,,
Magnesic ,, ...	3·150 ,,
,, bromide	0·070 ,,
,, sulphate	2·066 ,,
Calcic ,, ...	1·331 ,,
Ammonic chloride	0·0004 ,,

And traces of magnesic nitrate and carbonate, lithium chloride, ferrous carbonate and several other salts.

A series of media were made up of peptone beef broth gelatine to each of which 1½ per cent. of one of the following salts was added :—sodic chloride, potassic chloride, magnesic chloride, magnesic sulphate, calcic sulphate, calcic carbonate, and ammonic chloride. There were thus seven distinct media, all containing the same nutritive materials but differing only in the salt added. These tubes were inoculated from an active culture of photobacterium phosphorescens. The tube first to phosphoresce, after an interval of four days at room temperature, was the one containing ammonic chloride. The sodic chloride tube followed directly after, and a few hours after this the potassic chloride tube. The same experiment was repeated, and the inoculation was made from a very bright culture so that the track of the needle was plainly phosphorescent, and the same result was arrived at. In all cases where a soluble chloride was used some phosphoresence was visible, but all the other salts failed to produce any.

It was, therefore, concluded that a medium containing either or all of the chlorides occurring in sea water might be

expected to give the best results, and this was found to be the case. The best medium obtained after several experiments was by taking 1 litre of beef broth peptone gelatine and adding to it

Sodic chloride	26·5	grammes
Potassic ,,	·75	,,
Magnesic ,,	3·25	,,

the relative quantity of these salts being the same as in sea water. Gelatine media with fish broth, such as herring or mackerel instead of beef broth, were also tried, but they did not give such good results; in fact, without the addition of some chlorides the fish broth failed to give a trace of phosphorescence with any of the organisms. The gelatine medium mentioned is the one that has been most largely used in the present investigation, and it has been found that the organisms retain their vitality and their power of phosphorescing for very long periods when cultivated on it.

An agar medium does not seem to give such uniformly good results, although some of the organisms grow well on an agar gelatine medium made as follows:—

Fish or beef broth	950	cub. c.
Gelatine	70	grammes
Peptone	5	,,
Agar-agar	5	,,
Sodic chloride	26·5	,,
Magnesic chloride	3·25	,,
Potassic ,,	·75	,,

An agar medium is, however, hardly needed, as the majority of these organisms lose their power of phosphorescing if the temperature exceeds that at which an ordinary gelatine medium remains solid.

The most satisfactory liquid medium is made by boiling herrings, mackerel or codfish, in water, to which has been added the three chlorides in the proportions before mentioned, and adding to this, after it has been filtered, ½ per cent. of peptone. A liquid culture medium is, however, rarely satisfactory, as the supply of oxyen is so limited, but it becomes useful when aeration is resorted to as described later. In making any fluid media for photogenic bacteria it is necessary that after the final sterilisation the tube or

flask should be thoroughly shaken up, so that a certain amount of oxygen may be taken up to replace that driven off during sterilisation.

The determination that no medium is favourable to the growth of phosphorescent bacteria which does not contain some soluble chlorides indicates that these salts are in some way intimately bound up with the light-emitting power of these organisms, and it has appeared to us more satisfactory to use media in which the substances which do not favour phosphosescence are as far as possible eliminated than to use those having sea-water as a base, and which, therefore, contain several salts which can only hinder its production. All media should be neutral or slightly alkaline. Various fish are suitable as soils for the growth of these organisms, the most useful being herring, mackerel or codfish. They are best prepared by taking a slice off the side of the fish and cutting it into narrow strips, so that they slide easily into a test-tube. The tube should have at the bottom a little cotton wool thoroughly saturated with sea water or salt and water. These can be placed in the steam steriliser for about twenty minutes on two successive days, and are then ready for use.

A peptone water medium is rarely satisfactory, as further organic nutritive material than peptone alone contains is necessary. The nutrition of photogenic organisms has been studied by Beyerinck,[1] who states that most of these organisms require more than one organic compound for their growth. In some cases we have also found that to be so, although in many instances it has been observed that light has been emitted when peptone alone has been present. The addition of a small percentage of sugar is stated by Beyerinck to favour light production, and a large percentage to hinder both growth and light function. The latter statement appears to be well founded, although we have been unable to verify the former, except in the case of photobacterium caraibicum.

All photogenic bacteria are halophil, and the medium for their nutrition must contain at least 2 per cent. of saline

[1] *Centralbl. f. Bakter.*, vol. viii.

matter. The increase of this amount beyond 3 per cent. does not seem in most cases to have any beneficial result, and if the quantity is increased to 5 per cent. and over both the growth and the light emitted are reduced until with 7 per cent. or over, reproduction entirely ceases.

In using plate cultures for the isolation of photogenic organisms it is sometimes difficult, when inoculating tubes from small colonies, to identify them after having recognised them in the dark. It has been found possible, however, after examining plates in total darkness to still discern the phosphorescent colonies if, instead of taking the plates into white light, they are illuminated by the light from a photographic ruby lantern. The red glass chosen should be as nearly as possible monochromatic; especially should it transmit no green rays. It will by this arrangement be found quite easy to identify phosphorescent colonies whilst having sufficient light in which to make inoculations on culture media.

All photogenic bacteria require a supply of free oxygen, and are best grown in the dark. The characteristics and morphology of the organisms studied will now be considered.

Photobacterium phosphorescens, also known as photobacterium phosphorescens gelidus. This is the most widely distributed and best known of all photogenic bacteria; we have therefore taken it as a typical example of this group of organisms, and have devoted some time to the study of its morphological and biological peculiarities. It occurs on the bodies of nearly all dead fish, and is most easily obtained from dead herring or mackerel, on which it makes its appearance within twenty-four hours of the death of the fish. It also occurs on meat or bread when an appreciable quantity of saline matter is present. The light emitted by it is of a brilliant green colour, and its presence is easily ascertained by this, as no other organism has such a characteristic phophorescence.

This organism is sometimes regarded as a putrefactive one, but it appears to take no part in the decomposition of fish or meat, and its growth is retarded and ultimately entirely stopped by the development of other putrefactive

organisms. On the contrary, its presence on fish might almost be regarded as an indication that the fish is still in a condition fit for human consumption, as it will develop when the fish is kept in an ice chest, and when, therefore, the conditions have not been favourable for the growth of putrefactive bacteria.

It is a short thick bacillus varying from 1·5 μ to 4 μ in length, and almost appears to be a diplococcus when subdivision occurs. It is extremely pleomorphic, and is particularly susceptible to changes in the culture media. On ordinary gelatine it remains fairly constant in form, but rather elongated forms are sometimes seen in young cultures. (Fig. 1.) When, however, the organism is allowed to grow for several generations on this medium its appearance alters greatly. A three days' growth from an old culture is seen in fig. 2, in which the organism has become much shorter and thicker, and is very inconstant in shape. Vacuolation is also seen and involution forms are common. A capsule also appears to develop at this stage. On a salted gelatine medium the organism is considerably shorter in young cultures, a twenty-four hours' growth at 18° C. being seen in fig. 3. In older cultivations, however, on this medium a radical change seems to take place. Owing to the large quantity of saline matter in this medium the organism becomes quite yeast-like in form and develops a large vacuole. Its appearance is shown in fig. 4. Its size is most variable, many of the organisms appear to alter entirely and have no constant form. This change has been termed plasmolysis by Fischer. It can, however, be restored to its normal shape and size by being grown again on ordinary gelatine or a fluid medium.

On fluid media the organism assumes a much longer and thinner form. On peptone sea-water its form is seen in fig. 5, and it appears to retain this form constantly on any fluid medium. In the hanging drop it is seen to be motile, and does not form chains. A fairly copious growth is seen in twenty-four hours at 20° C. It has a single flagellum which is difficult to stain, but it can be demonstrated by van Ermengem's osmic acid silver process after prolonged treat-

ment. It stains easily with the usual dyes, figs. 1, 2, 3 and 5 having been stained with methyl violet, and fig. 4 with fuchsine, but it is decolorised by Gram. On tube cultivations it exhibits a thick white growth which is confined almost to the area of inoculation. In fluid cultures the growth appears to be fairly equal throughout the liquid, no surface pellicle being formed. The phosphorescence is confined to the surface unless the tube is agitated or a current of air passed through. In plate-cultures the colonies are small, and in three days are seen as white semi-transparent colonies rarely exceeding ·25 to ·5 mm. in diameter. They have a sharply defined edge. Microscopically they are yellowish in colour and have a central zone, with a lighter coloured border. Surface colonies phosphoresce brightly, but those in the depth do not. It grows very slowly on potato, and spore formation has not been observed.

According to Beyerinck, who has very carefully studied the nutrition of photogenic bacteria, this organism grows well on a maltose-gelatine medium and requires at least 3·5 per cent. of saline material. He also states that at least two organic nutritive bodies are required for them to become phosphorescent, a peptone-like body and a carbohydrate, peptone alone or amides alone not being sufficient. It has not been possible to entirely verify this statement, as the organism will grow and produce light on peptone seawater, although its activity on this medium is of but short duration, and its growth and light production are somewhat hindered. In successive cultures, however, with all light-producing bacteria, their power varies so considerably that it is sometimes very difficult to determine whether a variation which occurs is due to the medium or to some other altered condition, to which sufficient regard has not been paid. It has therefore often been found necessary to repeat experiments many times before any definite result could be obtained.

It is a facultative aerobic organism, but a supply of free oxygen is absolutely necessary for it to exhibit its light-giving properties. It grows on all the ordinary culture media and does not liquefy gelatine. It grows luxuriantly

and phosphoresces brilliantly on the gelatine medium with soluble chlorides added, as previously described, and also develops well in fluid cultures when a suitable proportion of these salts is present. The addition of ½ per cent. asparagin to the medium slightly increased its light-giving powers. It retains its power of producing light for long periods, even up to several months. On a medium containing no saline matter it grows very slowly, and exhibits no phosphorescence, as also occurs when the salts are in excess. The most brilliant results have been obtained when the salts are present in the quantities mentioned, and either an increase or decrease in these quantities results in a decrease of the phosphorescence.

This organism retains its power of emitting light when the amount of free oxygen present is small. If the culture tubes are placed in a large glass vessel containing alkaline pyrogallic acid, and the bottle sealed down, the small amount of oxygen which remains free is sufficient to enable the organisms to phosphoresce, but as soon as it exhausts the oxygen the light dies out. If however, the oxygen is entirely removed by filling the bottle and tubes with pure hydrogen, nitrogen, or carbonic acid gas, the organism still grows although very slowly, but no phosphorescence appears. This is not due to any specific action of these gases, as if after a week or ten days a small quantity of oxygen is let in, the organism commences to emit light. For these experiments Erlenmeyer flasks were used, so that as large a surface as possible was exposed to the particular gas. Although the oxygen flask resulted in the brightest culture in seventy-two hours, it did not phosphoresce as soon as the control flask, in which atmospheric air was used. The growth in oxygen is very rapid, but carbonic acid gas prevents growth entirely. Rapid growth does not necessarily result in a proportionately rapid production of phosphorescence.

Its optimum temperature is from 10° to 15° C., but, as previously stated, it can grow and phosphoresce at 0° C. Reproduction at this temperature, however, is slow, but the organism retains its vitality for lengthened periods, even

when considerably below this. If a phosphorescing culture is placed in a freezing mixture consisting of muriate of lime five parts and ground ice or snow four parts, it is reduced to a temperature of 40° C. No decrease in the light is seen until freezing of the medium takes place when the phosphorescence almost instantaneously ceases. Its function is entirely restored, however, if the culture is removed from the freezing mixture and placed at room temperature, when as the medium thaws the phosphorescence is seen to re-appear. The organisms do not seem to in any way suffer by this subjection to a low temperature, but emit light as freely as before. Their power of absorbing oxygen and therefore of emitting light does not seem to be hindered by a low temperature so long as nutrition is not stopped.

At temperatures above 20° C. its growth gradually decreases until at 27° C. it entirely ceases. It grows on fluid media, such as mackerel and cod-fish broth made with sea-water, but requires aeration. If grown in an atmosphere of pure oxygen the phosphorescence is extremely brilliant, the finest results being obtained by having a stream of oxygen passed through the culture medium if it is fluid, or over it if solid.

A culture medium consisting of cod-fish or mackerel broth to which no soluble chlorides or sea-water was added, gave negative results, thus showing that the organism does not depend on the fish for its supply of materials for light productions, although its growth in this instance is not greatly hindered. Cultures should be grown in darkness to obtain the best results, and to retain them in a phosphorescent condition they should be kept from exposure to daylight. If this is done the cultures remain phosphorescent for seven or eight weeks when kept at room temperature. If the tubes are exposed to daylight their power is lost in seven to ten days. Any extraneous source of light seems to have the effect of decreasing the light-giving power of the bacteria.

With the object of determining whether this organism would still remain photogenic if grown in various coloured

lights, three active tube cultures were taken and immersed in larger test-tubes containing respectively :—

(1) Saturated solution of bichromate of potash (which absorbs blue and violet, allowing green and red to pass).

(2) Ammonic sulphate of copper (which absorbs red and yellow, allowing blue and violet to pass).

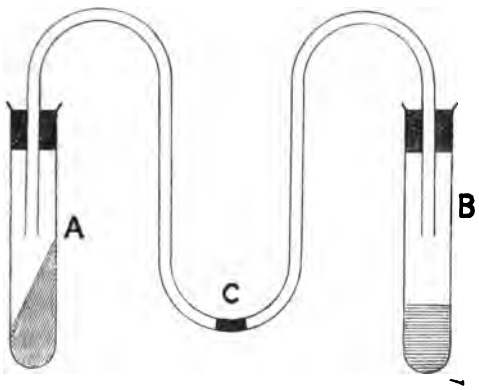
(3) Zetnow's solution (150 gr. cupric nitrate, 14 gr. chromic acid, water 250 c.c.), (which absorbs nearly all but a portion of green and the yellow).

The cultures were allowed to remain in a strong light which was filtered through these solutions for several days and gradually the phosphorescence decreased. No conclusion could be arrived at as to the action of any particular colour, as the organisms in each tube seemed to lose their light-giving power as quickly as when grown in white light. Therefore it was concluded that any extraneous source of light, whatever its nature, has the effect of decreasing the light-giving power of these organisms. This is probably due, however, to a general lowering of the vitality of the organisms themselves, owing to the well-known action of light on bacteria, although it might have been expected that the light of greater wave-length would have less action than those of short wave-length, as occurs with most other organisms which are affected by light.

The amount of light emitted by photobacterium phosphorescens varies directly as the rapidity with which oxygen is absorbed. It has already been stated that the organism can grow anaerobically but under this condition emits no light. If the culture medium is so altered that the production of light is hindered, the amount of oxygen absorbed is also less. To determine this point salted gelatine culture media were made up containing respectively 1 per cent. of maltose and levulose. These tubes were then inoculated, and when growing the tube with maltose emits rather less light than a control tube of salted gelatine only. The levulose tube emits very little light although the growth is apparently as rapid. To insure that the number of organisms should in each case be approximately equal, the tubes were inoculated from a fluid culture in which the organisms

were well-distributed, and one loopful was introduced into each tube.

These tubes were arranged as shown :—



The tube A contains the medium with the organisms growing on it. The tube B is equal in air capacity to the tube A, only having water in it to more easily regulate the capacity. These two tubes are then connected by a tube of small bore which is plugged with cotton wool at either end, and has in the bend at C a globule of mercury. Any increase or reduction in the amount of air in either tube would therefore result in the mercury moving towards the tube which has the relatively smaller quantity of air. It is obvious that the object of the two tubes is to establish a condition of equilibrium, so that changes of temperature would not be of any moment, provided they equally affected both tubes, which, of course, would be the case when enclosed in an incubator. Each of the three cultures mentioned were arranged in this way and the rapidity of movement of the mercury was noted. The control tube absorbed oxygen most rapidly, the mercury quickly moving towards the tube containing the growth. The maltose tube did not absorb oxygen so rapidly, and the levulose tube did so still more slowly. After several experiments this was determined to be quite constant, and demonstrated that the

rate of oxygen absorption and light emission are directly proportional.

Ozone has been shown by Professor Dewar and others to have the effect of causing phosphorescence in vacuum tubes under certain conditions. It was, therefore, thought that an atmosphere containing ozone might have some effect on photobacterium phosphorescens. To avoid the effect of nitrous fumes ozone was prepared in a Siemens ozonizer from pure oxygen. This was then slowly passed over an active culture of the organisms, an equally luminous culture being kept with it as a control. The first effect of the ozone was to cause a slight increase in the light emitted by the organisms, but this occurred only when the amount of ozone present was very small, it only being possible to detect its presence by the iodide of potassium test. On increasing the ozone the light immediately diminished, and was in a short time entirely stopped. The well-known bactericidal action of ozone is, of course, sufficient to account for the latter effect, but it is somewhat interesting that it should cause any increase in the phosphorescence when present in sufficiently small quantities.

The question whether the bacterial bodies themselves phosphoresce, or whether it is produced only by their metabolic products which are excreted by them, is a point of great interest and considerable difficulty. That the light is emitted by the product is the opinion held by Radziscewski, Quatrefages, Ludwig and Dubois; the latter even calls the phosphorescing substance "Luciferin." In higher marine animals which phosphoresce there does seem to be in some cases a secretion which is the source of the phosphorescence, and by analogy it might be argued that bacteria would in all probability be subject to the same conditions. However, it should not be overlooked that the secretions and even the excrement of some of these animals owe their phosphorescence to the presence of light-producing bacteria. Regarding, however, a bacterial cell as a simple protoplasmic body, it is quite possible that by the process of oxidation, which, as already shown, is so intimately bound up with the production of light, the organism itself becomes luminous.

This is a process quite analogous to the production of light by fire-flies.[1] "If you examine the luminous cell of the common fire-fly, you will find it filled with peculiar yellowish white granules, the whole cell reminding one of some actively secreting gland. These granules, by combining with oxygen brought in through the trachea and tracheal capillaries, which closely invest the cells from all sides, give out light. These granules are the product of metabolism, the result of a secretion process, due to the decomposition of the living substance of the cell; but, instead of being thrown out of the body, like some other products of secretion, they are consumed *in situ* by combining with oxygen brought in from without. As to the chemical nature of this material little is known; but it is a secretion of a fatty nature, which oxidizes readily in alkaline media. Phosphorus has nothing to do with the phenomenon." This phosphorescent product of fire-flies is able to produce light without the insect itself still carrying on its functions; as even after death it still continuous to phosphoresce, "although it owes its existence primarily to the life activity of the cell as a whole," and can be made to give out light or stop doing so at will simply by supplying it with or depriving it of oxygen. In bacteria, however, the light production is intimately associated with the life and cell substance of the organism. If a fluid culture of photobacterium phosphorescence is passed through a Berkefeld filter, the filtrate has no trace of phosphorescence, but the organisms remaining on the filter still emit light, and will continue to do so as long as there is sufficient material left in the pores of the filter for their nutrition. This, of course, cannot be regarded as conclusive evidence that the phosphorescence is exclusively a function of the bacterial cell, but at the same time it does not seem unreasonable to expect that, as the fluid culture medium itself is able to pass through the filter unaltered, the bacterial product also, if it existed and was soluble, might be able to do likewise. Again, the light can be stopped at any time by treating an artificial culture with any known bactericide, and

[1] Watase. "On the Physical Basis of Animal Phosphorescence."

the stoppage of light-production is proportional to the rapidity of action of the antiseptic substance. Bichloride of mercury (1 per cent. solution), formalin, permanganate of potash, ether, chloroform, phenol and other substances have the effect of stopping light-production in a shorter or longer time according to their rapidity of action on the vitality of the organism. This extinction of the light is not merely due to stoppage of the oxygen supply, as, if the culture is covered with distilled water, which has been in addition thoroughly boiled and which therefore contains no oxygen in solution, the light is very slowly affected; and even after its entire cessation a sub-culture on a fresh medium will result in a phosphorescent growth. This shows, therefore, that the vitality of the organism is not impaired by the want of oxygen, although its destruction immediately results in the stoppage of the light-production.

On the other hand, it is of course well known that a great number of bacteria have the power of excreting products, such, for instance, as chromogenic organisms, but light-production cannot be regarded as analogous to that. Heat and light are now known to be but variations of the same form of energy, the difference only being one of degree. There is probably, therefore, no radical difference between the production of heat, as the direct result of chemical change, in our bodies, and the emission of light by other living organisms. Differentiation between the two is only due to the fact that our central nervous system is stimulated in a different manner by each, although all light waves have a greater or less heating effect. The difference of wave-length between red and violet light is only one half; that is, the waves causing the red sensation are only twice as long as violet. Comparing this with sound waves, the whole of the visible spectrum is included in one octave, and yet in this octave there is nearly all included that gives rise to the sensation either of light or heat. Certainly, radiant heat is of somewhat greater wave-length than the visible red, but the difference is smaller. As will be shown later, the light emitted by bacteria rarely, if ever, reaches the

region of the red, or extends in the other direction to the violet. Therefore it does not seem unreasonable to conclude, until some further evidence is available, that the light production is as much an accompaniment of the organism's vitality, as heat production is in higher animals.

Spectroscopically the light emitted by the photogenic organisms examined by me is confined to a very small portion of the visible spectrum; never extending into the ultra-violet or infra-red. Visually it only includes the green and blue, and photographically it extends very slightly further into the violet. The economic value of such a light source is obvious, and it is a matter of great practical importance to determine its method of production.

These organisms do not seem to be directly acted upon by an electric current of low potential. If a current of about eight or ten volts is passed through a fluid culture, no alteration is visible in the phosphorescence until the gas given off is of sufficient volume to act directly on the organisms. At the kathode, therefore, as hydrogen is evolved, the phosphorescence diminishes; while at the anode, owing to the production of oxygen, no decrease occurs.

These experiments have all been carried out with cultures of photobacterium phosphorescens, as that organism retains its phosphorescence for long periods in artificial cultures, and can also be obtained from fish at any time. The other organisms have been found to be so similar to this one, as to the general conditions of light-production, that in nearly all cases where experiments have been repeated with them the results have been found to be similar, and need not therefore be detailed.

During a visit to the laboratories at the Marine Biological Association of the United Kingdom at Plymouth, where the opportunity offered itself of observing a large number of the phosphorescent marine animals which occur round our coasts, an organism which cannot be identified with any of those already mentioned was isolated, which will for purposes of identification be referred to here as *Photobacterium Liquefaciens Plymouthii.* Some polyzoa

were obtained by dredging at considerable depth outside Plymouth breakwater, and a sterilised needle was passed over the surface of these. Plates were then made, and in four days several phosphorescent colonies were seen. Plates made from sea-water, also obtained from a depth, resulted also in the growth of two or three of these colonies in some of the plates. Their number in sea-water could not have been large, as several loopfuls of water to a tube of gelatine only resulted in two or three colonies. Their number per c.c. could not be definitely determined, as some plates would develop several while many other plates had none at all. The chief characteristic of the organism is that it liquefies gelatine much more rapidly than any of the other organisms mentioned. Its phosphorescence also does not occur until the growth is about four days old when kept at room temperature, and then, especially in plate cultivations, lasts for but a short time. In some plates it only retains its phosphorescence for a few hours, but develops it again on inoculation into fresh medium. Successive inoculations on artificial media containing at least 2 per cent. of sodic chloride seems to result in the organisms having an increased power of phosphorescing, and cultures many generations old retain their power for some days. The organism grows well on an ordinary gelatine medium, but its phosphorescent power is best seen on the salted gelatine medium already described. In plate cultivations the liquefaction is very rapid and soon involves the whole. In gelatine complete liquefaction occurs in four days at 20° C. It grows on fluid media, such as herring or beef broth, and will phosphoresce on peptone sea-water. The growth is fairly abundant in forty-eight hours and sinks to the bottom of the tube. In all fluid media the phosphorescence is of short duration. It will also grow slowly on fish, but does not phosphoresce for long. A gelatine medium containing $\frac{1}{2}$ per cent. glucose is not favourable to the phosphorescence, and $\frac{1}{2}$ per cent. levulose stops it entirely. Maltose in quantities of not more than $\frac{1}{2}$ per cent. does not hinder or increase light-production. It grows abundantly on agar. Old cultures retain their vitality for several weeks, and if

inoculations from them are made on fresh media, the organism grows and recovers its phosphorescence.

When grown on ordinary gelatine it appears microscopically as a short thick rod; sometimes coccus forms appear, and very occasionally filamentous forms are to be seen which extend to a considerable length. It does not generally exceed 2 μ in length, and in breadth is about 1·5 μ. (Fig. 7.) On salted gelatine the rod is slightly longer and somewhat comma-shaped. (Fig. 8.) In cultures more than forty-eight hours old involution forms occur, the organism becoming larger and inconstant in shape. Vacuoles are also occasionally seen but are not so marked as in photobacterium phosphorescens. On fluid media the organism retains its coccus form and short chains are seen. In the hanging drop it is motile. When cultivated on fish, such as herring, it is rather longer in form and slightly curved. Preparations from a maltose-gelatine cultivation, which phosphoresced, show the organism in its typical form, but on levulose-gelatine, which stops phosphorescence, it becomes very inconstant; sometimes being a short thick rod, and sometimes an elongated thin one. The organism stains easily with the usual dyes, but it is decolorised by Gram. It has very long fine flagella which extend all round the organism to as much as six or eight times its length. These can be demonstrated by Van Ermengem's method, but require prolonged treatment. The light it emits is of a bluish-white colour. It will not grow on potato, and spore-formation has not been observed.

I have not been able to identify this form with any of the known species described.

I now proceed to give an account of the other photogenic bacteria that I have had an opportunity of studying.

Photobacterium Balticum, also called vibrio balticus by Beyerinck, an organism isolated from the waters of Kiel Harbour by Fischer where it occurs in considerable numbers. It grows best at 15° C. to 20° C., and can even grow at 0° C. It is a motile bacillus and will liquefy gelatine somewhat rapidly, although not nearly so rapidly as photobacterium liquefaciens Plymouthii. It does not usually entirely

liquefy a gelatine tube for five or six days. On plate cultivations the colonies appear in about forty-eight hours. They gradually liquefy the gelatine and have the appearance of bubble-like hollows in which the growth sinks to the bottom. The colonies remain phosphorescent for several days and emit a bluish-white light of considerable intensity. The liquefaction does not spread far. The colonies are microscopically granular in appearance with a well-defined serrated edge, and in forty-eight hours measure ·25 to ·4 mm. in diameter if grown at 20° C. It will grow on ordinary gelatine, but requires a larger percentage of saline matter to become brightly phosphorescent. It grows and phosphoresces well on the salted gelatine medium already described. The growth on gelatine is not characteristic; it grows but slowly on agar, and neither maltose nor glucose favour its phosphorescence. It grows freely on fluid media, such as fish broth or peptone beef broth, but requires a supply of free oxygen. If a fluid culture is not agitated or aerated the phosphorescence only appears at the surface. No surface pellicle is formed, the growth extending throughout the liquid. It is very motile in herring or mackerel broth, but is not so active on peptone sea-water. The organism stains well with all the usual anilin dyes, but is decolorised by Gram. Spore formation has not been observed, although in some preparations vacuolation is seen which closely resembles it. Microscopically the organism is very pleomorphic. On gelatine it grows as a short rod, frequently comma-shaped, which measures about 3 μ in length and ·5 to ·75 μ in breadth. It is very inconstant both in shape and size, however, and filaments are frequently seen which extend right across the field of the microscope when using a $\frac{1}{12}$th in. oil immersion objective (fig. 9). Spirillar forms are also common, and sometimes extend to considerable length. In older cultures involution forms appear which are much shorter and thicker, sometimes almost yeast-like in form. On peptone sea-water the organism is much smaller and appears as a rod. Curved forms are rarely seen, and the bacteria frequently aggregate into groups. (Fig. 10.) Peptone alone does not seem to afford

it sufficient nutrition to develop well, consequently it becomes attenuated in form, but on fish-broth it develops again into its characteristic shape, spirillar and comma forms being common.

On maltose-gelatine it is again smaller, and long filaments are rare. On levulose gelatine involution forms appear and the organism is most inconstant in shape. It possesses a single flagellum which is seen at one end of the organism extending to about its own length. The flagellum is easily demonstrated by Loeffler's method, and is frequently seen even in ordinary cover-glass preparations which have been well stained with methyl violet. By van Ermengem's method it is easily demonstrated and is frequently seen at the extremity of the spirilla. (Fig. 11.) In a hanging drop from an active fish-broth culture the flagellum can be seen even when *unstained*, and has an active wave-like motion propelling the organism through the liquid at a considerable speed.

Photobacterium indicum, also known as bacillus phosphorescens indicus, and vibro indicus. This organism was first isolated by Fischer from West Indian waters, by the ordinary plate cultivation method, and from this it derives its names. It is essentially a tropical bacillus and requires a temperature of from 22° to 30° C. for its growth. At a lower temperature than this its growth is slow, and if kept for long periods at room temperature it loses its vitality and soon dies. It liquefies gelatine rapidly and at 20° C. a tube is completely liquefied in about three days. In plate cultivations small phosphorescent whitish colonies are seen in about three days. The colonies appear as liquid depressions in which the growth is evenly distributed, and liquefaction does not usually spread sufficiently far to involve the whole plate. Under a low power they appear to have a sharply defined edge, and young colonies have a distinctly iridescent appearance, but this disappears after a short time as the growth thickens. They ultimately become a dirty yellow colour and the periphery is not well defined.

On salted gelatine considerable growth is apparent in forty-eight hours at 22° C., which is highly phosphorescent.

It is not characteristic in appearance, merely resembling any other liquefying organism, until the growth sinks to the bottom of the tube, when it seems to aggregate into brightly phosphorescent lumps. In still older cultivations complete liquefaction of the gelatine occurs and the growth forms a thick surface pellicle, which is phosphorescent for some time. The light emitted by the organism is bluish-white and closely resembles photobacterium balticum. On an ordinary gelatine culture medium it phosphoresces but slightly. On fluid media, such as fish-broth, it grows well and forms a surface pellicle, which is brightly phosphorescent. On peptone sea-water the growth is flocculent and phosphorescent only at the surface. On agar it forms a thin, whitish growth, which, if the medium contains the necessary saline material, is phosphorescent, although not so brilliant as on a suitable gelatine medium. It can grow slowly and in an attenuated form on potato, but does not phosphoresce. Spore formation has not been observed. It does not grow at all below 10° C. and is destroyed by a temperature above 50° C. Microscopically it is seen, when taken from an ordinary gelatine culture forty-eight hours old, as a medium-sized rod about three times as long as broad. Occasionally coccus-like forms occur, also involution forms, when the culture is more than three days old. A vacuole also appears in the rod when it is about to sub-divide. On a salted gelatine medium the organism is much more constant in shape and is seen as a rod measuring 1·5 to 3 μ in length and about ·75 μ in breadth, with slightly rounded ends. It also forms threads of considerable length. The addition of maltose to a salted gelatine medium does not favour the phosphorescence of the organism, and microscopically it results in a shorter, more rounded form, which does not stain so readily and in which involution forms frequently occur. In the presence of glucose it retains its typical form as on salted gelatine alone, except that chains occur occasionally. On agar the organism is considerably smaller and varies in size. Very long, thin forms also occur, and also short ones, which are almost like cocci. On fish-broth it appears slightly smaller and thinner, but no radical altera-

tion in its appearance occurs, as in the case of most photogenic organisms. It seems to form into clumps on fluid media, such as peptone beef-broth or fish-broth, but otherwise its appearance remains the same. On peptone sea-water occasional long, spirillum-like forms are seen. As might be expected from its motility, this organism is richly endowed with flagella. They are very fine, long appendages, which extend from it on all sides to several times its length. It resembles the typhoid bacillus in appearance, except that the flagella are finer. By van Ermengem's method they are shown well, but require careful treatment to obtain satisfactory preparations. (Fig. 11.)

It stains with the usual dyes, but is decolourised by Gram. A supply of free oxygen is necessary for its phosphorescence, and stoppage of the supply results in rapid extinction of the light. It is one of the most difficult of light-producing organisms to maintain in an active condition in artificial cultivations requiring to be frequently re-inoculated on to a fresh medium.

Photobacterium caraibicum.—Isolated from sea-water by Fischer. On plate cultivation the growth is slow even at 22° C. It grows more rapidly at 25° to 30° C., closely resembling photobacterium indicum in this respect, and may be regarded as a tropical bacillus. It grows on all the usual culture media. In plate cultivations, after about three days, small well-defined colonies appear which are slightly yellow in colour. They grow to about 1 mm. in diameter, and do not appear to increase in size after that. On ordinary gelatine, or on salted gelatine, its growth is not characteristic, being almost confined to the track of the needle. Very slow liquefaction of the medium occurs generally, but not invariably. It phosphoresces faintly on a gelatine medium containing ½ per cent. glucose. It grows well in fluid media, such as fish-broth or peptone sea-water, forming a surface pellicle, but does not phosphoresce on them. It grows very slowly on peptone beef-broth, but on salted agar its growth is abundant and phosphorescence occurs. It is motile in the hanging drop. Microscopically it is seen on ordinary gelatine as a very short thick rod, almost coccoid in form,

which is frequently vacuolated, and involution forms are common. On salted gelatine it is even shorter and quite coccus-like, but occasional long filamentous forms occur. Its appearance on glucose gelatine remains the same, but it lengthens slightly on agar, and the longer filamentous forms are of rather more frequent occurrence. It slightly increases in size on a maltose gelatine medium but otherwise remains unchanged. On fluid media it slightly lengthens in appearance and frequently appears to be capsulated. It stains easily with all the usual dyes. It possesses very feeble light-producing power in artificial cultivations, the light emitted being almost white.

Photobacterium hirsutum.— An organism isolated by Fischer. It rapidly liquefies gelatine and grows easily and rapidly on the usual media at 15° to 25° C. On plate cultures the colonies develop rapidly, and in three days at 20° C. a small thick circular growth is seen, surrounded by a large liquefied zone. The central spot only is phosphorescent and emits a bluish-white light. It grows and phosphoresces on glucose-gelatine but does not on maltose or levulose gelatine. A large and rapid growth occurs on ordinary agar. In liquid media it grows well and phosphoresces brightly on fish-broth, but not so well on peptone sea-water. In the hanging drop it is slightly motile. · Microscopically it appears as a rather small straight rod, about three times as long as broad, and on ordinary gelatine long filamentous forms are common. On salted gelatine the filaments are much longer, and involution forms occur. Spores also appear in cultures more than three days old. Maltose or levulose does not effect any change in its appearance. In fish-broth it is considerably shorter, the long filaments not being common, while on peptone sea-water it becomes a short bacillus of medium size. It stains easily by the ordinary method. Flagella have not been demonstrated.

Photobacterium tuberosum.—This organism is likewise described by Fischer, and is a slowly growing organism which causes slight liquefaction of gelatine. It does not phosphoresce on artificial media and quickly loses its vitality. It grows best on the salted gelatine medium already mentioned,

and is seen microscopically as a small straight rod, the appearance of which is not characteristic.

Photobacterium fischeri.—An organism closely resembling photobacterium balticum except that it does not liquefy gelatine quite so rapidly, and after being cultivated artificially for some time it loses its power of doing so altogether. It grows well on all the culture media mentioned, its optimum temperature being from 15° to 20° C. It emits a bluish-white light, but it does not retain its power of phosphorescing for lengthened periods. Microscopically, when grown on gelatine, it is seen as a rather small thin rod, frequently comma-shaped, and filamentous forms are common. It remains almost the same on fluid media except that the comma forms are more marked and long spirilla are frequent. It stains easily with all the usual dyes.

Photobacterium glutinosum (Fischer). — An organism which is both difficult to obtain and maintain in a phosphorescent condition in artificial cultivations. It grows slowly at 20° C., and slightly liquefies gelatine. It grows well on fluid media at 22° C. Microscopically it is a medium-sized rod, about three times as long as broad, and the organisms appear to lie parallel to one another, slightly resembling the diphtheria bacillus. Its appearance is not altered when grown on fluid media, except that it does not take up the parallel arrangement. It stains easily with all the usual dyes.

Photobacterium coronatum.—Another organism first isolated by Fischer. It grows well on the culture media mentioned, and rapidly liquefies gelatine. It is difficult to maintain in a phosphorescent condition in artificial cultivations. When cultivated on gelatine it is seen as a very small thin rod, its appearance not being characteristic.

Photobacterium degenerans.—Isolated by Fischer. It grows well on any medium suitable for the cultivation of other photogenic organisms and liquefies gelatine. It is a small rod of variable shape, sometimes being straight and sometimes comma-shaped. Very short forms also occur which resemble cocci. It stains easily by all the usual methods.

Photobacterium pflugeri, also known as bacterium phosphorescens (Pfluger) and bacillus phosphorescens gelidus (Forster). Its growth on gelatine is somewhat transparent, the growth apparently sinking into the surface of the gelatine as if there were slight liquefaction. The organism is closely related to, if not identical with, photobacterium phosphorescens already fully described—to which reference may be made.

Photobacterium javanense, — (Eijkmann). Another tropical bacillus, which requires a relatively high temperature for its development. It grows well on the usual culture media and does not liquefy gelatine. It is a micrococcus of medium size, and should hardly be classed with the photobacteria. It does not maintain its light-producing power on artificial media.

Photobacterium luminosum (Beyerinck) also known as bacillus argenteo-phosphorescens liquefaciens (Katz) and vibrio luminosus. An organism which grows rapidly at room temperature on culture media already mentioned, and liquefies gelatine rapidly. It is very variable in shape, sometimes appearing as a short rod, but long filaments are common. Small coccus-like forms also occur on gelatine media. It is generally about 2·5 μ long and about ·75 μ broad. It is motile in hanging drop specimens, and stains well with all the usual dyes. Its phosphorescence is easily lost in artificial cultures.

The phosphorescent bacteria appear to be non-pathogenic with the possible exception of Giard's bacillus.

The general conclusions we have come to are the following:—It is probable that various forms of these organisms that have been described as different species are identical or are closely allied varieties. The forms that are probably identical are mentioned above along with their synonyms.

A marked feature of the phosphorescent bacteria is their tendency to undergo involution and the pleomorphism that occurs. These are possibly due to the artificial culture media which do not represent the natural conditions under which they live. The best culture medium was found to be

nutrient gelatine with the addition of soluble chlorides in the relative proportions in which they occur in ordinary sea-water. · A supply of free oxygen is necessary for the exhibition of phosphorescence, and when the bacteria are grown in an atmosphere of oxygen the phosphorescence is extremely brilliant. An extraneous source of light diminishes the light-giving power of the bacteria. The rate of oxygen absorption and light emission is directly proportional. It was experimentally proved that when the production of light is hindered the amount of oxygen absorbed by these organisms is also less. Traces of ozone cause a slight increase in the amount of light emitted. It is possible that by the processes of oxidation, which are so intimately bound up with the production of light, the bacteria become luminous. The phosphorescent principle does not pass through a Berkefeld filter, and all agents which affect the life of the organisms affect likewise the production of light. We have not been able to identify the photobacterium liquefaciens plymouthii with any of the hitherto described species. As will be seen from this paper, the pleomorphism of these organisms is most marked, a given form presenting at one time a rod shape and at another time assuming the form of a spirillum, whilst mixed forms are not infrequent. This renders their exact classification a matter of great difficulty, and I am not yet in a position to say how far certain of these organisms are to be considered as genuine bacilli or spirilla.

The opportunities I have had, through the courtesy of Mr. Allen, of the Marine Biological Association, Plymouth, for examining specimens of phosphorescent marine animals, showed in every instance that the phosphorescence was not in any way dependent on the action of bacteria and that it ceased with the death of the animal.

I am indebted to Dr. Macfadyen, at whose suggestion the research was undertaken, for much help and advice. A short bibliography is attached of the papers referred to. I hope to continue these observations in a future communication.

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DESCRIPTION OF FIGURES.

Fig. 1.—Photobacterium Phosphorescens. × 750.
Fig. 2.— ,, ,, × 750.
Fig. 3.— ,, ,, × 750.
Fig. 4.— ,, ,, × 750.
Fig. 5.— ,, ,, × 750.
Fig. 6.— ,, ,, × 750.
Fig. 7.— ,, Liquefaciens Plymouthii. × 750.
Fig. 8.— ,, ,, × 750.
Fig. 9.— ,, Balticum. × 750.
Fig. 10.— ,, ,, × 750.
Fig. 11.— ,, ,, × 750.
Fig. 12.— ,, Indicum. × 750.

Figs. 1 to 6 were photographed with a $\frac{1}{10}$ inch objective by Leitz, using no ocular, the necessary magnification being obtained by camera extension. Figs. 7 to 12 were taken with a 3 mm. apochromatic objective, aperture 1·40 and projection ocular 2.

FIG. 1

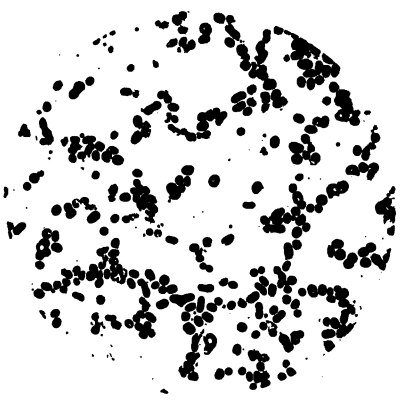

FIG. 2

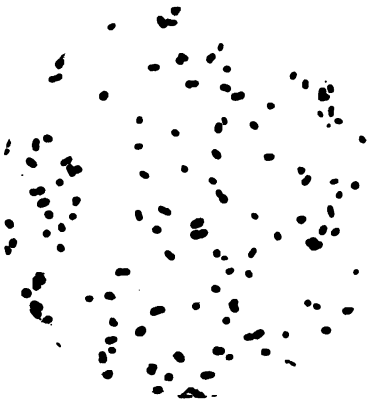

FIG. 3

FIG. 4

FIG. 5

FIG. 6

Photoprint, Bale & Danielsson, Ltd.

FIG. 7

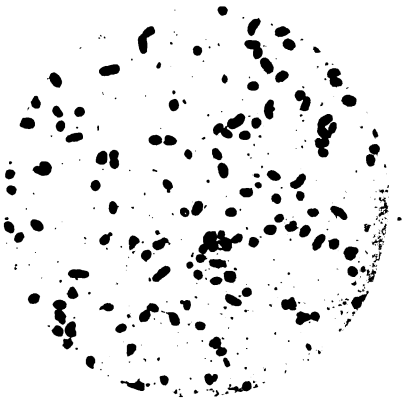

FIG. 8

FIG. 9

FIG. 10

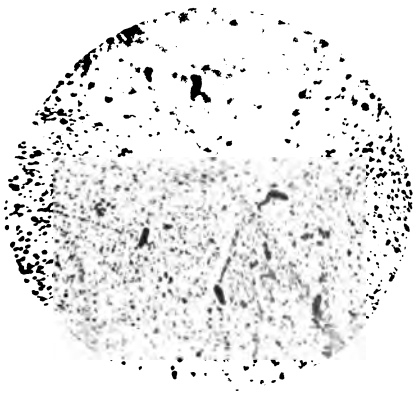

FIG. 11

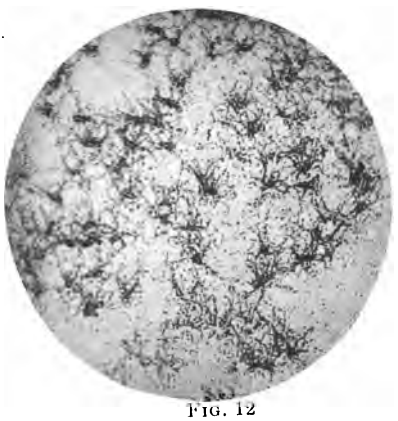

FIG. 12

Photoprint, Bale & Danielsson, Ltd.

THE PATHOGENICITY OF THE PSEUDO-DIPHTHERIA BACILLUS AND ITS RELATION TO THE KLEBS-LOEFFLER ORGANISM.

BY ALFRED SALTER, M.D. (LOND.)

SINCE the first descriptions of the pseudo-diphtheria bacillus were published by Loeffler (1887) and Hoffmann (1888) two diametrically opposite opinions as to the relationship of this organism to the Klebs-Loeffler bacillus have been held by different investigators. On the one hand, the view championed by Roux and the French school maintains that the so-called pseudo-species is merely an attenuated form of the true diphtheria organism, which has suffered modification both in its morphological and biological characters as the result of environmental influences. On the other hand, we have the hypothesis originally enunciated by Loeffler, which places the organism in a category quite distinct from the genuine and virulent species, and assumes that it has no causal relation to the diphtheritic processes but that it plays merely a saprophytic or accidental *rôle* in anginal affections.

In the last volume of these Transactions the question was considered at some length in the paper of Drs. Hewlett and Knight, and the points of resemblance and difference between the two microbes were subjected to a searching review. The principal distinctive features upon which these and other authors have relied for diagnostic purposes have been (1) the staining reaction with methylene blue, (2) the production of acid, and (3) the pathogenicity towards guinea-pigs. The differential colouration with methylene blue in the case of the Klebs-Loeffler bacillus is now regarded by prac-

tically all bacteriologists as insufficient in itself to mark off the two species one from the other in an absolutely unequivocal manner. Even Neisser's method of double staining with acid blue and vesuvin has met with much hostile criticism,[1] and, indeed, Hewlett and Knight themselves state[2] that "in a number of cultures it is possible to obtain a series of organisms which form a connecting chain between the Klebs-Loeffler and the pseudo-bacillus." Any value which may have been attached to the method of distinguishing the former organism by its power of elaborating acid in neutral media during the first hours of culture, and of the latter by its production of alkali under the same circumstances, has now been swept away by the description by Kurth[3] of a special variety of the pseudo-bacillus (B. pseudo-diphthericus acidumfaciens), which manufactures acid like the Klebs-Loeffler bacillus from the very commencement of its development. Neisser[4] also has met with some pseudo-species which exhibit the same phenomenon. The only certain test with which we are left, therefore, is the one of animal experiment; and in this connection all writers are agreed that the pseudo is totally non-virulent to guinea-pigs, or at most is capable of inducing only a slight and transient local œdema.[5] According to Schanz,[6] at the present time Loeffler himself holds that the one reliable distinction (durchgreifende Unterschied) between the true and false organism lies in the power of secreting toxin.

The non-pathogenicity of Hoffmann's bacillus is, however, only relative, as it has been shown by L. Martin and by Simonin and Benoit[7] that it is sufficiently active to kill sparrows and a small exotic bird called the Calfat. Experiments were, therefore, undertaken with the hope of exalting the virulence of the pseudo-bacillus to the extent of ulti-

[1] *Vide* esp. Schanz, *Münch. Med. Woch.*, March 15, 1898, p. 333.
[2] These TRANSACTIONS, 1st series, p. 32.
[3] *Zeitschr. f. Hyg.*, bd. xxviii., 1898, p. 431.
[4] *Ibid.*, bd. xxiv., 1896, p. 443.
[5] Spronck, *La Semaine Med.*, Sept. 20, 1897, p. 353, and Glucksmann, *Zeitsch. f. Hyg.*, bd. xxvi., 1897, p. 445.
[6] *Deutsch. Med. Woch.*, Aug. 18, 1898, p. 522.
[7] *Compt. Rend. de la Soc. de Biol.*, Jan. 8, 1898, p. 24.

mately rendering it pathogenic towards guinea-pigs; and the results, which will be detailed in the following pages, speak very strongly in favour of the view of the racial identity of the two organisms.

Previous Attempts to Convert the Non-Virulent into the Virulent Form.

Roux and Yersin[1] in 1890 found that feebly virulent diphtheria bacilli injected into guinea-pigs along with the streptococcus could be markedly exalted in activity by this procedure; but they failed to render pathogenic bacilli which were totally destitute of virulence to begin with. Aronson,[2] Funck,[3] Spronck,[4] Bardach,[5] and Martin[6] have all succeeded in increasing the initial virulence of diphtheritic organisms by successive passages through guinea-pigs, dogs, rabbits, &c.; but Trumpp[7] in 1896 was the first to accomplish the transformation of a completely harmless organism of the pseudo-type into one capable of killing guinea-pigs. His *modus operandi* consisted in simultaneously inoculating a dose of the culture with a small quantity of diphtheria toxin. After three passages in this manner through guinea-pigs he found that the pseudo-bacillus had acquired lethal powers, and now killed animals with all the typical lesions of experimental diphtheria.

Hewlett and Knight[8] in 1897 by a method of alternate growth on blood serum and in bouillon, followed by passage through a guinea-pig, also succeeded in converting a non-virulent short bacillus of the Hoffmann class into a pathogenic organism exhibiting all the characteristic features of Klebs-Loeffler group.

[1] *Ann. de l'Inst. Pasteur*, t. iv., 1890, p. 409.
[2] *Berlin. Klin. Woch.*, June 19, 1893, p. 592.
[3] *Manuel de Sérumthérapie antidiphtérique*, Bruxelles, 1895, p. 18.
[4] *Ann. de l'Inst. Pasteur*, t. ix., 1895, p. 763.
[5] *Ibid.*, p. 40.
[6] *Ibid.*, t. xii., 1898, p. 42.
[7] *Centralb. f. Bakt.*, bd. xx., 1896, p. 721.
[8] *Loc. cit.*, p. 25.

Investigation of the Pathogenicity of Hoffmann's Bacillus towards Birds.

Nineteen separate strains of pseudo-diphtheria bacillus, isolated and supplied to me by Dr. B. A. Richmond, of the Brook Fever Hospital, Shooter's Hill, were examined in the first place as to their pathogenicity towards different species of small birds. It was found that all the nineteen were capable of killing members of the finch and bunting families (*Fringillidæ* and *Emberizidæ*), though when tested quantitatively the respective organisms showed nearly as much difference in activity towards any given bird as do different stocks of Klebs-Loeffler bacillus towards the guinea-pig. Similarly, the various species of birds displayed the most striking differences in susceptibility. The goldfinch (*Carduelis elegans*), the chaffinch (*Fringilla cœlebs*), the canary (*Serinus canarius*), the siskin (*Carduelis spinus*), and the hedge-sparrow (*Accentor modularis*), proved to be the least resistant, whilst the thrush (*Turdus musicus*), the blackbird (*Turdus merula*), and others were apparently immune. The following table exhibits the relative susceptibility of the species examined, the most feebly resistant being placed first :—

Goldfinch (*C. elegans*)	Every one of the 19 strains proved pathogenic to this species.
Chaffinch (*F. cœlebs*)	Every one of the 19 strains proved pathogenic to this species.
Canary (*S. canarius*)	18 out of 19 strains pathogenic.
Siskin (*C. spinus*)	3 strains only tested; all pathogenic.
Hedge-sparrow (*A. modularis*)	8 strains tested; 6 pathogenic.
Robin (*Erythraca rubecula*)	,, ,,
House-sparrow (*Passer domesticus*) ..	14 strains tested; 8 pathogenic.
Greenfinch (*Coccothraustes chloris*) ..	13 strains tested; 2 pathogenic.
Yellow Hammer (*Emberiza citrinella*) ..	13 strains tested; 1 pathogenic.
Brambling finch (*Fringilla montifringilla*)	3 strains tested; 1 pathogenic.
Linnet (*Linota cannabina*)	11 strains tested; 1 pathogenic.
Blackbird (*T. merula*)	Totally insusceptible.
Thrush (*T. musicus*)	,, ,,
Hawfinch (*Coccothraustes vulgaris*) ..	,, ,,
Starling (*Sturnus vulgaris*)	,, ,,
Pigeon (*Columba livia*)	,, ,,

Exaltation of Virulence of the Pseudo-Diphtheria Bacillus.

Having thus ascertained the relative immunity of different avian species, attempts were made to augment the virulence of the organism by successive passages at first through the more susceptible, afterwards through the comparatively immune, and finally through the completely resistant birds. It was soon found that as the result of a couple of passages through a chaffinch any given stock was rendered capable of causing every linnet inoculated to succumb; whilst after two more passages through the latter bird, the thrush and blackbird were no longer proof against the organism. Similarly, three passages through the goldfinch elevated the virulence of one strain to such an extent that it became capable of killing the blackbird in forty-eight hours, though previously it had not caused even temporary illness. Finally, it was ascertained that repeated passage through almost any susceptible bird rendered the pseudo-diphtheria bacillus pathogenic to the guinea-pig. Details of one experiment will sufficiently describe the procedure adopted:

A bacillus was employed which was originally obtained from a case of post-scarlatinal diphtheria. It possessed the following characteristics:—

(1) A short, fairly plump, regular rod, 1·5 μ in length, readily coloured in a uniform manner with methylene blue or bleu de Roux, and showing no differential or polar staining, *i.e.*, morphologically a typical Hoffmann's bacillus. (*Vide* fig. 1.)

(2) Colonies on blood serum were of a pearly-white colour.

(3) In neutral broth it always produced an alkaline reaction from the first few hours of culture.

(4) 10 c.c. of a forty-eight-hour broth culture, in which were emulsified two whole serum-cultures, failed to kill a 230 gr. guinea-pig.

(5) A filtered seven-day alkaline bouillon culture was inoffensive to guinea-pigs in doses as large as 20 c.c.

The organism was, therefore, entirely "non-pathogenic" in the ordinary sense, and would undoubtedly be classed as a typical pseudo-diphtheria bacillus.

Half a serum culture (scraped off with a platinum loop) was suspended in about 1·0 c.c. of a forty-eight hour old bouillon culture.[1] This was injected with the most minute aseptic precautions into the left pectoral muscle of a goldfinch. The bird appeared quite vigorous during the first twenty-four hours, and was eating well. On the second day it became much quieter than normal, and remained motionless on its perch, hardly eating anything. At the end of forty-eight hours it kept its head persistently under its wing, its feathers were ruffled, and its respiration was slow, deep, and laboured. External stimuli, such as stroking the bars of the cage, which ordinarily caused it much agitation, now had no effect. It took no food whatever on the third day, and died in just under ninety-six hours from the date of inoculation. At the *post mortem*, three hours after death, cultures on blood-serum and in bouillon were made from the site of injection. Around the latter there were marked ecchymosis and pallor of the muscle, together with definite turgidity and swelling. On microscopical examination bacilli, apparently identical with the original organism inoculated, though perhaps a trifle longer, were found in and around the local lesion. Beyond a few subcutaneous and subpleural petechiæ no other abnormal appearances were detected in the body. Hæmorrhagic reddening of the suprarenal capsules—so distinctive and pathognomonic a feature in diphtheria-infected guinea-pigs—was absent. A few organisms were present in the heart blood, and still more in smear-preparations from the liver.[2]

The cultures yielded pure growths of the organism, and a twenty-four hour old specimen stained with bleu de Roux from a blood-serum tube is shown in fig. 2. This culture

[1] Subsequent experiments showed that it is not necessary to employ nearly such large quantities of virus in the case of the goldfinch.

[2] It appears to be the rule rather than the exception for a certain number of organisms to find their way into the general blood-stream whenever a small bird is infected with a large dose either of the diphtheria or pseudo-diphtheria bacillus.

we shall call (A). 1 c.c. of a broth subculture made from (A) was now injected into a second goldfinch. The creature exhibited the same symptoms as goldfinch 1, and died in a little over forty-eight hours. *Post mortem* the lesions resembled those seen in the first bird, but cover-slip preparations from the pectoral muscle showed that many of the bacilli were definitely longer than the original stock, some indeed presenting an appearance quite transitional between the latter and the "short" Klebs-Loeffler bacillus. (*Vide* fig. 3.) The organism was easily recovered in culture (B) from the second bird.

A loopful of serum culture (B), suspended in 1 c.c. of broth, was next injected into a third finch. Death occurred on the third day, and in this subject the whole of the pectoral muscle on the side of the inoculation was of a yellowish-white colour, the fibres being very friable and obviously degenerated, whilst the substance was mottled with minute punctate hæmorrhages. The suprarenals were of the normal colour. Photographs of a cover-glass preparation from the local lesion are shown in fig. 4, whilst fig. 5 indicates the appearance seen in culture (C).

A small loopful of (C) was then injected into goldfinch 4, with the result that death followed within forty-eight hours. Preparations and cultures (D) are shown in figs. 6 and 7. The bacilli have assumed the typical short Klebs-Loeffler form, and give differential staining and polar coloration with methylene blue.

5 c.c. of a forty-eight hour broth culture of (D) plus one whole serum culture of (D) were at this stage injected into a guinea-pig of 250 grs. weight. The animal suffered a large local reaction, followed by necrosis, and was ill for several weeks, but ultimately recovered. It was obvious, therefore, that marked exaltation in virulence had already occurred, as a much larger dose of the original stock was unable to excite such symptoms.

A tiny loopful of culture (D) was now injected into goldfinch 5. The animal died in thirty-six hours, and from the local lesion perfect specimens of the diphtheria bacillus were obtained. (Fig. 8.) 5 c.c. of broth culture, plus the

whole of a serum culture recovered from this bird, and given to a 260 grs. guinea-pig, killed it on the fourth day with all the usual and well-known symptoms of experimental diphtheria. Its suprarenals showed the pathognomonic reddening (though those of goldfinch 5 did not), and there were also present a marked gelatino-serous local reaction and an extensive pleural effusion. A second pig, inoculated with 5 c.c. of a broth culture obtained from the last animal, succumbed on the sixth day, and the bacilli recovered from the abdominal wall were found to have been further transformed into the long Klebs-Loeffler type. This, however, is not an uncommon result of passage of any diphtheritic organism through a rodent, the increase in length being definite though variable.

The pathological effects of the exalted bacillus could be completely neutralised by antitoxic serum, not even a transient œdema resulting around the site of inoculation. Its true nature and specificity were thus proved beyond doubt, though as a further confirmation the reaction changes in neutral bouillon were examined. Two tubes made from the same brew of broth were planted side by side, the one with a sub-culture of the original organism, the other with one derived from goldfinch 5. After sixteen hours' incubation the liquid in the first tube had become distinctly alkaline, whilst that in the second was equally distinctly acid.

The conversion of the non-pathogenic into the pathogenic form was repeated with three other strains of pseudo-organism, one of which was kindly furnished me by Dr. Hewlett. There are certain difficulties in details of technique, especially in relation to ensuring pure unmixed infections in the highly susceptible subjects, and also in recovering the organism from the tissues after death. With intramuscular injections the actively multiplying bacilli are often very locally situated, although stray organisms are not infrequently met with in the blood; and unless the exact spot be hit upon and many tubes planted, the culture media may remain sterile. On account of the thinness of the integument in small birds, and of the absence of much subcutaneous cellular

tissue, it is not easy to give a purely hypodermic, as distinguished from an intramuscular, inoculation. If this can be effected, however, then the pseudo-diphtheria bacillus is found to induce a large local gelatinous or gelatino-hæmorrhagic effusion, exactly comparable to that occurring in the guinea-pig.

As regards the absence of suprarenal lesion, I have found that the sign is systematically wanting in small birds, even when the latter have been infected with the Klebs-Loeffler bacillus. In a series of fourteen experiments, conducted upon five sparrows, one goldfinch, two chaffinches, three greenfinches, two yellowhammers, and one thrush, suprarenal reddening was not once observed, although in all cases a typical Klebs-Loeffler bacillus was employed as the infecting agent. Consequently, the absence of this *post-mortem* sign, in birds succumbing to the exalted pseudo-diphtheria organism, cannot be taken as evidence of its non-alliance or non-identity with the genuine virus.

Further, just as certain strains of the Klebs-Loeffler bacillus are more virulent towards the guinea-pig than others, so the different stocks of pseudo-bacilli vary amongst themselves as to their activity towards any given species of bird. For example, 20 mgm., 10 mgm.,[1] and half a serum culture respectively, represented the minimum lethal dose of three separate organisms for the hedge-sparrow; whilst 2 mgm. and 10 mgm. were the fatal doses for the chaffinch of two other strains.

Conclusions.

There are to be met with diphtheritic organisms of every grade of virulence. The weakest, known as Hoffmann's, or the pseudo-diphtheria bacillus, and representing the most attenuated form of the Klebs-Loeffler bacillus, is capable of killing only certain highly susceptible small birds of the finch tribe. Organisms of a slightly higher degree of virulence can kill

[1] These experimental quantities were measured out by platinum loops of different sizes, which were identical in construction with that described by Washbourn and Eyre in *Journ. of Path. and Bacter.*, vol. v., 1898, p. 14, and used by them for a similar purpose.

other and more resistant small birds of the bunting family. Yet others, still more active, can cause larger birds (Merulidæ) to succumb, whilst the most virulent of all can kill certain of the rodents, such as the guinea-pig.

The Products of Metabolism of the Pseudo-Diphtheria Bacillus.

If the pseudo-diphtheria bacillus is grown for from seven to ten days in neutral or alkaline beef-broth, and the liquid is then filtered through a Pasteur-Chamberland candle, a germ-free product is obtained which causes no detectable ill-effects when injected into guinea-pigs, even in doses as large as 25 or 30 c.c. Quantities as small as 1·0 c.c. or 1·5 c.c., however, of the filtrate will rapidly kill chaffinches and goldfinches, control birds treated with corresponding doses of the original sterile broth remaining unaffected. We may, therefore, legitimately speak of a "toxin" of the pseudo-diphtheria bacillus.

A physiological analysis of this toxin shows that it contains bodies identical with, or allied to, certain of the constituents of the true diphtheria poison, known as "toxoids." Like the latter substances the products of the metabolism of Hoffmann's bacillus are innocuous to guinea-pigs, but none the less possess in a marked degree the power of neutralising or "fixing" antitoxic serum. These facts were demonstrated in the following way :—

A diphtheria toxin, prepared in the ordinary manner, was standarised against a glycerine test-serum obtained direct from Prof. Ehrlich, and containing one antitoxic unit (*ein Immunisirungeinheit*) per c.c. The precise quantity of toxin which could neutralise one unit of serum was thus accurately defined, as is shown in the accompanying protocol.

Animals injected with quantities of the toxin-serum mixture containing more than ·32 c.c. of toxin died, whilst those receiving doses containing ·32 c.c. or less recovered. There must thus have been present in the latter mixtures an excess of antitoxic substance.

(T = Toxin. IE = An antitoxic unit. † = Death.)

	Weight.	Dose received.	Result.
Guinea-pig 1 ..	250 gr.	·35 c.c. T + 1 IE	† 3rd day.
,, 2 ..	255 gr.	·34 c.c. T + 1 IE	† 3rd ,,
,, 3 ..	252 gr.	·33 c.c. T + 1 IE	† 4th ,,
,, 4 ..	255 gr.	·32 c.c. T + 1 IE	Recovered.
,, 5 ..	258 gr.	·31 c.c. T + 1 IE	,,
,, 6 ..	250 gr.	·30 c.c. T + 1 IE	,,
,, 7 ..	245 gr.	·29 c.c. T + 1 IE	,,

A second series of guinea-pigs was now treated with similar solutions, containing in addition, however, varying amounts of a filtered broth culture of the pseudo-diphtheria bacillus.

(Ps. T. = Germ-free filtrate of the pseudo-diphtheria bacillus.)

	Weight.	Dose received.	Result.
Guinea-pig 8	260 gr.	·32 c.c. T + 1 IE + 1 c.c. Ps. T	† 3rd day.
,, 9	250 gr.	·32 c.c. T + 1 IE + 0·5 c.c. Ps. T	† 3rd ,,
,, 10	245 gr.	·32 c.c. T + 1 IE + 0·25 c.c. Ps. T	† 4th ,,
,, 11	255 gr.	·31 c.c. T + 1 IE + 0·5 c.c. Ps. T	† 4th ,,
,, 12	248 gr.	·30 c.c. T + 1 IE + 0·5 c.c. Ps. T	† 5th ,,
,, 13	252 gr.	·30 c.c. T + 1 IE + 0·25 c.c. Ps. T	† 8th ,,
,, 14	250 gr.	·29 c.c. T + 1 IE + 0·5 c.c. Ps. T	Recovered.
,, 15	255 gr.	·28 c.c. T + 1 IE + 0·5 c.c. Ps. T	† 6th day.
,, 16	240 gr.	·27 c.c. T + 1 IE + 0·5 c.c. Ps. T	Recovered.
,, 17	240 gr.	·26 c.c. T + 1 IE + 0·5 c.c. Ps. T	,,

It is quite clear from these experiments that the filtrate of a pseudo-diphtheria bacillus culture, though harmless in itself to guinea-pigs, possesses marked anti-toxin fixing properties. It exhibits an affinity for the serum, greater,

indeed, than the true diphtheria toxin, otherwise none of the animals (8 to 17) would have died. In these experiments the toxin-antitoxin mixture was always prepared first, and was allowed to stand for several minutes before the pseudo-filtrate was added. In the cases where the pigs died the filtrate must have possessed the capacity of breaking up the neutralised combination, liberating the toxin, and taking its place, thus leaving an excess of free poison sufficient to kill the animal. It is certain, then, that this power of the pseudo-bacillus product of seizing and "fixing" the anti-toxin molecules must be far greater than that of the true toxin, and to such a substance Ehrlich[1] has given the name of "protoxoid."

He has shown that under certain circumstances this body exists in ordinary diphtheria toxin, and the fact that it is now proved to be a common product of both the diph-theria and the so-called pseudo-diphtheria bacillus is yet another argument in favour of the identity of the two organisms.

[1] *Klin. Jahrbuch*, bd. vi., 1897, heft 2, p. 316.

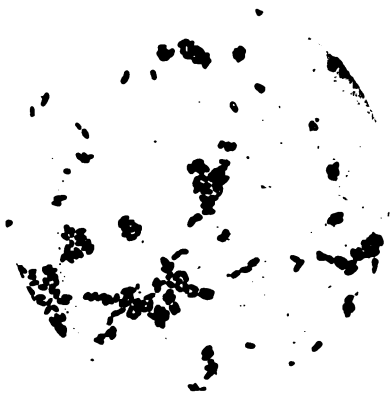

FIG. 1

FIG. 2

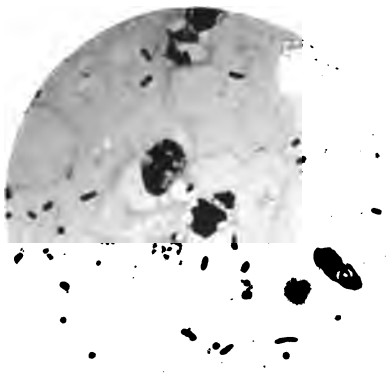

FIG. 3

FIG. 4

FIG. 5

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FIG. 6

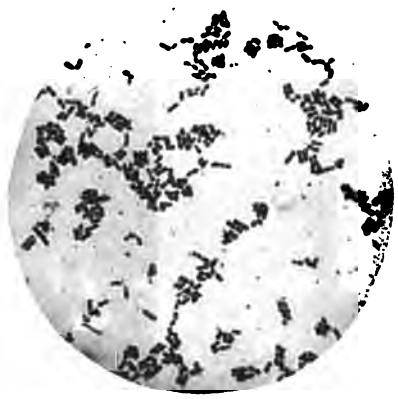

FIG. 7

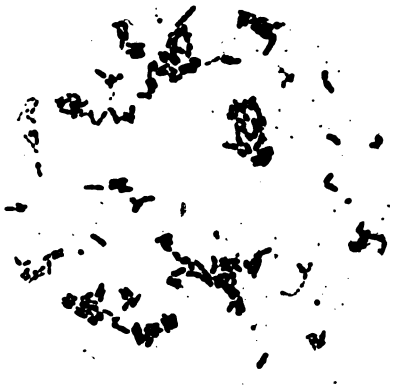

FIG 8

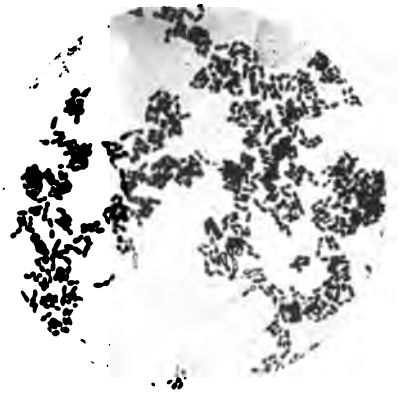

FIG. 9

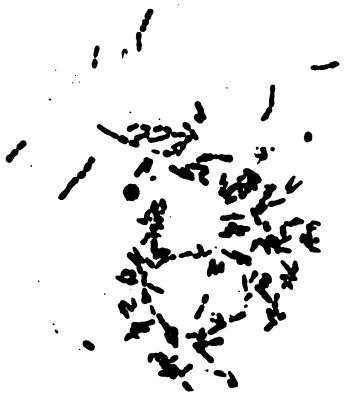

FIG. 10

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DESCRIPTION OF FIGURES.

Fig. 1.—Twenty-four-hour culture on Loeffler's serum of pseudo-diphtheria bacillus.

Fig. 2.—Twenty-four-hour blood serum culture of pseudo-diphtheria bacillus after passage through goldfinch 1.

Fig. 3.—Smear preparation from local lesion of goldfinch 2, showing transition forms.

Fig. 4.—Cover-slip preparation from local lesion of goldfinch 3. Typical short Klebs-Loeffler bacillus.

Fig. 5.—Twenty-four-hour blood serum growth after passage through goldfinch 3.

Fig. 6.—Cover-slip preparation from local lesion of goldfinch 4.

Fig. 7.—Twenty-four-hour blood serum culture after passage through goldfinch 4.

Fig. 8.—Twenty-four-hour blood serum culture after passage through goldfinch 5.

Fig. 9.—Twenty-four-hour blood serum culture after passage through blackbird.

Fig. 10.—Twenty-four-hour blood serum culture after passage of pseudo-diphtheria bacillus through guinea-pig, preceded by five passages through goldfinch.

THE FERMENTATION OF SUGARS BY BACILLUS COLI COMMUNIS AND ALLIED ORGANISMS.

Part I.

BY ARTHUR HARDEN, M.SC.

THE following experiments were undertaken with the object of examining the products of the fermentation of various sugars by Bacillus coli communis and allied forms, in the hope of finding some definite test of diagnostic value for the discrimination of the very numerous varieties of the coli group which have been described.

It was at once found that the action of the organism on sugar is largely modified by the nature of the nutritive medium supplied. This fact has already been observed by Péré and others.

The products of the action of bacillus coli communis on glucose are acetic acid, alcohol, lactic acid, carbonic acid and hydrogen, the last two being evolved in the gaseous state. The attention of investigators has been chiefly directed to the nature of the lactic acid produced, and it has been found that this differs both when different organisms are cultivated in media of the same composition and when sub-cultures of the same organism are cultivated in media of different composition. Péré (*Société de Biologie*, May, 1896, and *Ann. Inst. Past.*, 1898, xii., 63) has found that the bacillus coli communis, isolated from the intestine of an adult, produces lævo-lactic acid, whilst the organism isolated from the intestine of a child at the breast produces either dextro- or lævo-lactic acid, according to the nature of the nitrogenous nourishment. Thus, in presence of 15 grammes of peptone and 50 grammes of

glucose per litre, the latter organism produced a mixture of the inactive with the dextro-rotatory acid. On the other hand, in the same solution to which 1 gramme of phenol had been added, or in presence of only 1·5 grammes of peptone per litre, or in a solution in which the peptone had been replaced by 10 grammes of ammonium sulphate and 10 grammes of ammonium phosphate per litre, the lævo-acid or a mixture of this with the inactive acid was formed.

A similar result was obtained (*Ann. Inst. Past.*, 1893, vii., 737) with a bacillus obtained from the intestine of the rabbit. On the other hand, the organism isolated from the

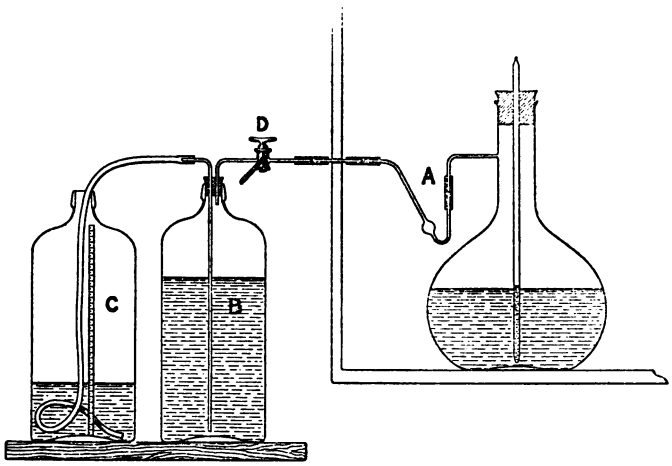


FIG. 1.

intestine of an adult gave lævo-lactic acid both in presence of peptone and of ammoniacal salts.

Still more recently Hugounenq and Doyon (*Ann. Chim. Phys.* 1898, xv. 145), by cultivating an organism, of which the origin is not stated, in a solution containing per litre 5 grammes of peptone, 67 grammes of glucose, and 0·7 gramme each of ammonium tartrate and potassium nitrate, together with inorganic salts, obtained a mixture of the inactive with the dextro-acid.

The results quoted in the present paper are derived from experiments in which the nutritive media were com-

posed of ordinary beef broth and peptone in various proportions. Particular attention was paid in each case to the gases evolved during the action, since it seemed possible that some simple method of diagnosis might be based on a measurement of the gases evolved.

The experiments were therefore carried out in the following way:—One litre of the solution was placed in a flask (fig. 1) provided with a side tube and an indiarubber stopper through which passed a straight glass tube leading to the bottom of the flask. The side tube and vertical tube were plugged with cotton wool and the flask then sterilised in the usual way. The side tube was then attached to a piece of bent tubing (A) on which a small bulb was blown near the bend. A drop of mercury was placed in the tube and served to seal the apparatus and prevent diffusion, whilst at the same time it readily allowed gases to pass out of the flask. After inoculation, which was effected by removing the stopper and introducing a loopful of the culture, the air of the flask was displaced by nitrogen. This gas was chosen because the gaseous products of the decomposition of sugar are carbonic acid gas and hydrogen, with the determination of which nitrogen does not interfere. If the experiment be commenced with the space above the liquid in the flask full of air, a partial absorption of the oxygen occurs, and this interferes with the accuracy of the measurement of the volume of gas evolved.

The nitrogen employed to displace the air of the culture flasks was prepared from the air by the method proposed by Berthelot. This consists in absorbing the oxygen by means of metallic copper in presence of ammonia. Two 10-litre bottles (A and B, fig. 2) provided with a tubulure near the base, are connected by a long piece of indiarubber gas tubing attached to glass tubes passing through indiarubber stoppers placed in the tubulures. One of the bottles (B) is about half filled with bright copper turnings, and its mouth is fitted with an indiarubber stopper through which passes a glass tube bent once at right angles and connected with an indiarubber tube provided with a screw clip (C). The second bottle (A) is filled with a solution of ammonia

(one of strong ammonia to three of water) and the ammonia solution is allowed to pass into the bottle containing the copper turnings to a depth of four or five inches. The screw clamp is then closed and the bottle shaken for a few minutes. The oxygen is rapidly absorbed, cupric oxide being formed which dissolves in the ammonia, whilst practically pure atmospheric nitrogen is left. The gas must then be washed through dilute sulphuric acid in order to remove ammonia, and it is also advisable to pass it through some solution capable of absorbing oxygen, so as to remove any

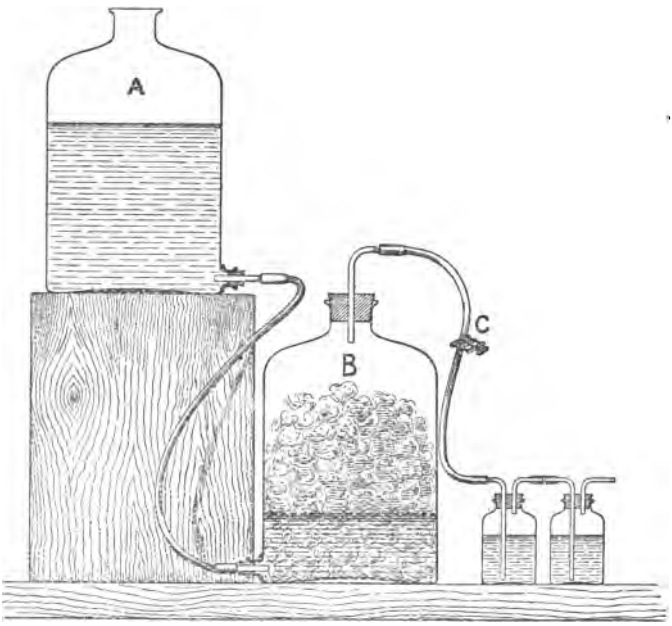


FIG. 2.

which may be left owing to insufficient exposure to the copper. The most efficient substance to employ is chromous chloride, but as this is somewhat troublesome to prepare, it will probably be found more convenient to use alkaline pyrogallic acid. The bottle containing the ammonia solution is then raised, and the pressure of the liquid drives out the nitrogen from the other bottle. At the close of the

experiment the tube C is disconnected from the washbottles, and the position of A and B reversed. The ammonia solution flows back into A, whilst air enters B. The clamp is then closed and B well shaken. After about ten minutes the whole of the oxygen is removed and the apparatus can be used again.

After passing nitrogen for about one to two hours, the flask is removed and the long vertical tube sealed off at a constriction previously made near the top. It is then placed in an incubator (fig. 1), the side of which is pierced by a brass tube, with which the tube A is connected by india-rubber tubing. The apparatus for collecting and measuring the gas is connected with the other end of the brass tube. It consists of a Winchester quart bottle (B) fitted as an aspirating bottle and provided with a long piece of india-rubber tubing passing to the bottom of a second bottle, graduated in volumes of 100 c.c. on a piece of paper pasted to the glass. On the tube between the flask and the collecting bottle is placed a three-way tap (D), by means of which samples of gas can be withdrawn either directly from the flask or from the collecting bottle. The collecting bottle is filled with saturated brine, on the surface of which a little oil is poured to prevent absorption of carbonic acid gas. Direct experiment showed that a mixture of carbonic acid gas and air could be preserved over this liquid for a considerable time without undergoing any perceptible alteration in composition.

About 100 c.c. of brine are placed in C, and the connecting rubber tube is also filled with brine so that the volume of gas evolved can be measured by that of the liquid displaced.

During the period of incubation the flask was agitated at frequent intervals in order to secure the neutralisation of the acid produced, and the volume of the liquid displaced was read off, the measuring bottle being raised or lowered until the surface of the liquid in it was at the same level as that in the collecting bottle.

As soon as about two litres of gas had been collected a sample of about 500 c.c. was taken for analysis. The

remainder of the gas was swept out through the three-way tap by raising the measuring bottle, and the apparatus then arranged as before for the collection of a fresh quantity of gas.

At the close of about fourteen days the flask was removed from the incubator, and a culture made on agar, which was examined and in every case found to give the usual tests for normal B. coli communis. The solution was then measured, and aliquot portions removed for the estimation of the various constituents.

(1) *Residual glucose.*—This was estimated by Pavy's method of titration with an ammoniacal copper solution. The gravimetric method of heating with Fehling's solution and filtering off the cuprous oxide is inadmissible, on account of the production of ammonia by the action of the alkali of the Fehling's solution on the nitrogenous matter present in the solution.

The end reaction with Pavy's solution is not very sharp, and hence the estimation of the sugar cannot be carried out with great accuracy. The best results are obtained by diluting the solution to be tested with four volumes of alcohol and testing the clear filtrate.

(2) *Free ammonia and total acids.*—50 c.c. of the liquid were boiled for some time with precipitated calcium carbonate, the distillate being received in standard acid and the ammonia estimated by titration. The amount produced was invariably very small. The liquid was then filtered from the excess of calcium carbonate, made alkaline with ammonia and precipitated with ammonium oxalate. The resulting calcium oxalate was filtered off, washed, and weighed as lime.

(3) *Volatile acids.—Alcohol.* An excess of oxalic acid was added to 500—800 c.c. of the clear liquid, and the calcium oxalate allowed to settle. An aliquot portion of the clear solution was then taken (400 to 700 c.c.) and distilled. The whole of the alcohol passes over in the first 200 c.c., and this quantity was therefore collected separately. The distillation was continued until only about 100 c.c. of the liquid remained, and this was then distilled in a current

of steam until the total distillate amounted to about 2 to 2·5 litres.

It was found to be practically impossible to obtain a neutral distillate in this manner, and special experiments showed that this was due to a very slow volatilisation of the lactic acid present. This is clearly shown in the following experiment: 100 c.c. of dilute sulphuric acid were distilled with steam in the apparatus usually employed for the distillation of the volatile acids. 250 c.c. of distillate were collected and were found by titration to be neutral, thus showing that no acid was being carried over mechanically, 3·5 grammes of pure crystallised inactive zinc lactate were then added, and the distillation continued. Three successive quantities of 250 c.c. of the distillate each required 0·25 c.c. of normal alkali for neutralisation. The error thus introduced therefore amounts to about 1 c.c. of normal acid per litre of distillate.

The total amount of volatile acid was estimated by titrating the whole of the distillate with normal alkali in presence of phenolphthalein.

The portion of the distillate containing the alcohol was separately titrated, and was then distilled in a flask connected with a Wurtz fractionating column, 50 to 100 c.c. of distillate being collected. The amount of alcohol in this was estimated by determining the specific gravity.

The neutralised liquid containing the volatile acids was evaporated to small bulk, and was then acidified with dilute sulphuric acid and distilled with steam until the distillate was neutral. An aliquot portion of the distillate was boiled with barium carbonate, and the resulting solution evaporated to dryness. The barium salt was then dried at 130°, weighed and converted into sulphate, from the weight of which the average composition of the acids was calculated. The remainder of the distillate was again neutralised and evaporated to small bulk. One-sixth of the sulphuric acid necessary for the complete liberation of the fatty acids was then added and the liquid distilled in a current of steam until the distillate became neutral. The distillate, which should contain the acids of highest molecular weight, was

treated with barium carbonate as described above. The further examination of these acids was not carried out in every experiment, since numerous analyses showed that practically the only product was acetic acid.

Thus, in a typical experiment, 700 c.c. of distillate were obtained, of which 150 were treated with barium carbonate. The barium salt obtained weighed ·4158 gr. and yielded ·3831 gr. of barium sulphate. Calculated for barium acetate, ·3800.

The remainder required 12·2 c.c. of normal caustic soda

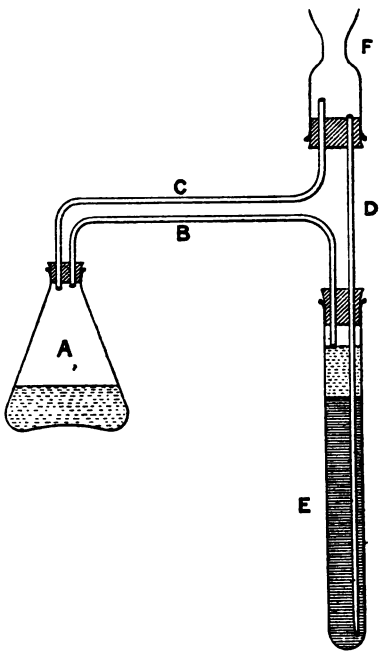


FIG. 3.

for neutralisation. To the concentrated solution 2 c.c. of normal acid were added, and the whole treated as described above. The barium salt obtained weighed ·2043 gr., and yielded ·1879 gr. of barium sulphate. Calculated for barium acetate ·1867. The whole of the acid present is, therefore, acetic acid.

Lactic acid.—The residue from the steam distillation was measured, and an aliquot portion taken for the estimation of lactic acid. This was effected by extracting with ether, removing the latter by evaporation, boiling the extract with water and calcium carbonate, filtering from the insoluble calcium oxalate, and estimating the dissolved calcium.

Since lactic acid is difficult to extract from aqueous solution, and the nutrient solutions tend to form an emulsion with the ether when shaken up by hand, it is convenient to employ some form of continuous extraction apparatus. A modification of that described by Foerster (*Chem. Ztg.*, 1898, 22, 421) has been found very suitable, and can readily be set up. An Erlenmeyer flask (A, fig. 3) is provided with a cork, through which pass two tubes, bent twice at right angles, one of which (B) passes downwards through an india-rubber stopper into the extraction tube (E), and the other (C) upwards into an inverted globe condenser (F). A third tube (D), drawn out at its lower end, passes from the condenser to the bottom of the extraction tube (E). The flask (A) contains the ether, and is heated on the water-bath. The ether vapour passes by the tube (C) into the condenser, where it is condensed to liquid, and flows down the vertical tube (D), from the end of which it rises through the liquid to be extracted, and finally passes back into the ether flask by the tube (B).

If care be taken at the commencement of the extraction that there is no sudden ebullition of the ether, very little frothing occurs, and the apparatus may be left without attention for many hours.

In order to ascertain the nature of the lactic acid produced, the greater part of the residue from the fatty acids was extracted with ether, as described above. The extract was then boiled with water and zinc oxide, and the solution, which was almost invariably brown, decolorised by animal charcoal. The zinc salt was then allowed to crystallise from the concentrated solution, and finally dried in the air. A portion was then submitted to analysis, and a second portion examined in the polarimeter.

The organisms employed for all these experiments were

supplied to me by Dr. Macfadyen, who was also kind enough to test the purity of the growths obtained in the various experiments. They were two in number, and were both of the strictly "normal" type of bacillus coli communis, derived from the digestive tract of an adult.

The following table gives the complete results obtained in two experiments made with sub-cultures of the same organism. Solution A consisted of beef broth made by boiling lean minced beef with twice its weight of water, filtering and adding to 1 litre, 20 grammes of pure glucose and 10 grammes of chalk. Solution B contained 10 grammes of Witte's peptone, 20 grammes of pure glucose, 10 grammes of chalk and 2 grammes of calcium phosphate.

	A	B
Sugar used..	13.42 g.	20 g.
Alcohol	1·70.	2·57.
Lactic Acid	6·6.	7·97.
Volatile Acids (in c.c. of Normal acid)	36·7.	62·8.
Total gas, under atmospheric conditions	4010 c.c.	6500 c.c.

These results are typical of the action of bacillus coli communis on glucose in beef broth and peptone water respectively.

The decomposition of the sugar is invariably more complete in the peptone solution than in the beef broth, and moreover, a further difference exists between the proportions in which certain of the products are formed. This is brought out in the following table, in which the amounts of the products per gramme of sugar decomposed are stated.

The lactic acid from A yielded inactive zinc lactate, $Zn(C_3H_5O_3)_2 + 3H_2O$.

0·7715 gramme lost 0·1393 gramme of water at 110°.
0·6318 gramme of the residual anhydrous zinc lactate yielded 0·2127 gramme of zinc oxide.

AMOUNT OF PRODUCTS PER GRAMME SUGAR.

	A	B
Volatile Acids, in grammes of acetic acid	0·16	0·19
Alcohol	0·12	0·13
Lactic Acid	0·49	0·40
Gas evolved at 0° and 760	272 c.c.	294 c.c.
Containing—		
Carbonic Acid liberated from the chalk ..	105 ,,	90·2 ,,
Carbonic Acid from glucose	82 ,,	91·8 ,,
Hydrogen	81 ,,	110 ,,

		Calculated.
Water	18·05	18·15
Zinc oxide in anhydrous salt	33·66	33·45

Examined in the polariscope, a solution of 0·5 grammes in 15 c.c. of water was found to be inactive.

The zinc lactate obtained from B., on the other hand, was dextrorotatory and consisted of a mixture of inactive zinc lactate with the dextrorotatory salt $Zn(C_3H_5O_3)_2 + 2H_2O$.

0·3099 gramme lost 0·0409 gramme of water at 110.°

		Calculated.
Water	13·2	12·9

The lactic acid produced in B. is therefore a mixture in which the lævorotatory acid predominates.

Throughout my experiments these results, as regards the nature of the lactic acid produced, have been confirmed. The addition of peptone to the beef broth appears not to

make any essential difference in the result. As a rule, when the experiment is carried out in the manner described above in a solution containing beef broth, a considerable amount of sugar is left undecomposed and an inactive lactic acid is produced. In a single case, however, a very vigorous action was obtained in a beef broth containing 10 grammes of peptone per litre, practically the whole of the glucose was decomposed, and in this case the zinc lactate was very faintly dextrorotatory, although the analysis showed that nearly pure inactive zinc lactate was present. It appears therefore that no sharp line can be drawn, but that, as a rule, the inactive acid is formed in beef broth solutions, whilst a small amount of active acid may be produced if the culture is a particularly vigorous one. The following experimental results illustrate this point. In all cases the solutions were made by boiling 1 lb. of lean minced beef with 1 litre of water and adding 20 grammes of glucose and 10 of chalk.

	Per Litre.			Per cent. of Water in Zinc salt.
	Sodium chloride.	Peptone.	Sugar left.	
1	5	10	6·5	18·09
2	5	10	6·6	18·11
3	—	10	10·1	18·15
4	—	10	8·4	18·16
5	—	10	15·0	18·18
6	—	—	6·6	18·10
7	—	10	0·4	18·09

Experiments 1, 2, 3, 4 and 5 were carried out with one organism, 6 and 7 with a different one, both derived from the fæces of an adult. No. 4 was only incubated for three days and No. 5 for only one day; all the others for at least ten days.

In the experiments made in peptone water, it was found that variation in the amount of peptone did not affect the kind of lactic acid produced, but that the vigour of the growth and the completeness of the action increased with increase of peptone. Experiments made with solutions containing 4, 7, 8·5, 10 and 20 grammes of peptone per litre all yielded mixtures of the inactive and lævorotatory acids.

As regards the *general nature of the chemical change*, no definite conclusion can be drawn from experiments in these media, since the organism exerts a very considerable action on the media themselves, which is, however, modified by the presence of glucose. A discussion of this question is therefore deferred until the completion of experiments

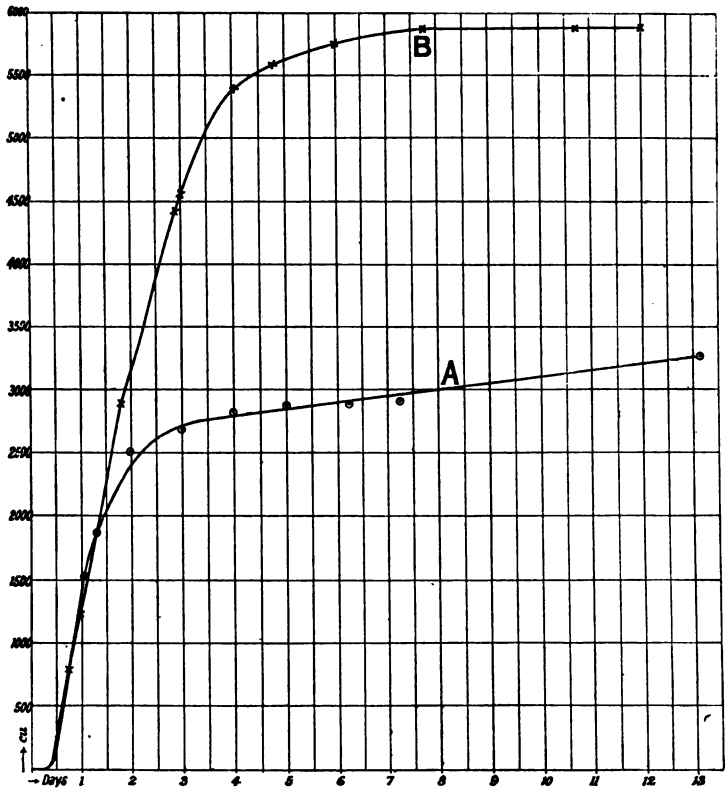


FIG. 4.

in which simpler media, such as ammonium salts, are being employed.

The gases evolved.—The evolution of gas from one litre of solution inoculated by a single loopful of an agar surface culture of the organism commences to be vigorous after nine to twelve hours, and is very rapid for about three

days, after which it usually becomes very slow. This is illustrated graphically in the curves (fig. 4), which show the volume of gas evolved at different periods during the course of experiments A and B. In both cases the evolution is very rapid at first, but in the medium containing beef broth the action soon begins to diminish, even although a considerable amount of sugar is still present in the solution, and then either ceases altogether or proceeds slowly for a considerable time.

The reason for this phenomenon, which has been observed in a large number of cases, has not yet been ascertained, but it is probably to be found either in the production of some toxic substance which hinders the further course of the change, or in the exhaustion of the available nitrogen of the medium. Experiments are at present being made on this point.

In the peptone water medium, on the other hand, a larger proportion of the sugar is usually destroyed, and the evolution of gas soon ceases. The smaller the amount of peptone present the less vigorous is the evolution of gas.

The volume of gas per gramme of sugar decomposed has been found to vary considerably in different cultures, but, as a rule, the volume evolved in peptone water is greater than that evolved in presence of beef broth. The gas evolved contains the carbonic acid liberated by the action of the acids produced from the sugar upon the chalk, and allowance must be made for this in calculating the amount of carbonic acid derived from the sugar. The solution in the flask also remains saturated with carbonic acid gas at 37° in an atmosphere composed of about 70 per cent. of carbonic acid. A direct experiment made by adding an excess of baryta water and titrating the excess showed that about 620 c.c. of carbonic acid are present in solution, and an allowance of this amount has been made in the following experiments.

The gas evolved varies in composition as the decomposition proceeds, the ratio of carbonic acid gas to hydrogen increasing steadily. This is largely, if not entirely, due to the absorption of the first portions of the carbonic acid

gas by the liquid. Thus in experiment B quoted above, the following samples of gas were taken :—

		CO_2	H_2	Ratio $CO_2 : H_2$
1	1220 c.c.	341·2	270	1·26 : 1
2	1670 c.c.	904·2	658	1·37 : 1
3	1670 c.c.	959·2	679·6	1·41 : 1
4	2020 c.c.	1201	819	1·47 : 1
	Dissolved	620	—	—
		4025·6	2426·6	1·66 : 1

Total gas evolved = 6500.
Found by analysis = 6453.
The difference of 47 c.c. is due to experimental error, or to a small quantity of nitrogen.

The following results may be taken as typical. 1 and 2 were carried out in a solution of 10 grammes peptone in 1 litre, 3 in beef broth, and 4 in beef broth containing 10 grammes of peptone.

	1	2	3	4
Sugar used	20	17·6	3·42	12·72
Total gas evolved	6500 c.c.	5720 c.c.	4010 c.c.	3440 c.c.
Gas at 0° and 760 mm. per 1 gramme sugar	294 c.c.	296 c.c.	272 c.c.	244·5 c.c.
Containing— Carbonic acid liberated by acids	90·2 c.c.	87·6 c.c.	105 c.c.	89·2 c.c.
Carbonic acid from sugar	91·8 c.c.	89·4 c.c.	82 c.c.	67·8 c.c.
Hydrogen	110·0 c.c.	116 c.c.	81 c.c.	83·1 c.c.
Ratio : $CO_2 : H_2$	1 : 1·2	1 : 1·3	1 : 1	1 : 1·23

Only in one case is the hydrogen exactly equal in volume to the carbonic acid, in the other three it considerably exceeds it. This difference may partly be accounted

for by the reducing action of the hydrogen on the medium, since it has been found in further experiments that the composition of the medium has a great effect on the volume of the hydrogen evolved.

In no case was marsh gas, or any other hydrocarbon found among the gases evolved.

In view of this result an experiment was made to ascertain the nature of the inflammable gas formed by the action of bacillus coli communis on nutrient gelatine. About 100 c.c. of nutrient gelatine were placed in a flask provided with a three-way tap, by means of which the gas evolved could be swept out by a current of carbonic acid gas. This was inoculated, the air displaced by carbonic acid, and the flask incubated at 37°, so that the gelatine became liquid and the gas was able to rise through it. After four days, the gas was swept out by carbonic acid gas and exposed to caustic potash. 8·7 c.c. were thus obtained and were found to have the following composition:

Hydrogen	5·7
Air	1·5
Nitrogen	1·5
	8·7

The air was, no doubt, partly derived from the carbonic acid gas employed and partly from the gelatine.

No carbonic acid gas was formed by the explosion of the gas with air, and hence no marsh gas was present. The inflammable gas is, therefore, hydrogen. It is described by Klein ("Micro-organisms and Disease," 1896, p. 125) as marsh gas.

The following conclusions may be drawn from the foregoing experiments. They apply only to the special organism employed, and cannot be considered as generalisations, since the number of organisms of different origin which has been examined is not sufficient to admit of a general statement.

(1) When grown anaerobically in a medium consisting of beef broth alone or in conjunction with peptone, the organism produces inactive lactic acid from glucose, but may, when a very vigorous growth occurs, produce a small amount

of the active, lævorotatory acid. The lactic acid produced amounts to about 50 per cent. of the weight of sugar decomposed.

(2) In media containing peptone, but without beef broth, the organism produces a mixture of the inactive acid with the lævolactic acid. This confirms the result of Péré, who obtained a similar result, with an organism of similar origin.

(3) The gas produced by the decomposition of the glucose consists of carbonic acid gas and hydrogen in proportions which vary between 1 to 1·3 vols. of hydrogen to 1 vol. of carbonic acid gas.

My thanks are due to Dr. Macfadyen for much advice with regard to the bacteriological portion of the work, as well as for the suggestion of the subject of research.

OBSERVATIONS UPON THE STRUCTURE OF BACTERIA.

BY SYDNEY ROWLAND, M.R.C.S.

THE microscopical study of the structure of protoplasm has now for many years attracted the attention of investigators, and the improved optical appliances at their command have greatly facilitated work in this direction. It is really only in the pursuit of such observations that one learns to appreciate the value of the finest powers of the microscope at our disposal, as well as to realise, it must be confessed, their limitations. This may account for the fact that a limited number of observers have devoted themselves to the microscopical study of living protoplasm, and that some, failing to make progress in such a difficult field, have left the investigation to the future and the improvement of optical instruments, which it may be expected to bring. Others, loth to relinquish this fascinating line of study, have fallen back on methods which, while facilitating observation, have never been free from the suspicion of producing the very structure which it was their object to demonstrate in the living cell. The first essential, I consider, in such an investigation is to ensure the active continuance of vital processes in the cell during the period in which it is under observation.

If, for example, a *paramœcium* be examined under natural conditions with a higher power of the microscope and allowed to die whilst under observation, an object-lesson of the first importance to all who are working at the structure of the living cell will be witnessed. The normal appearance of this organism is so familiar that it is hardly necessary to repeat its description. The point that now interests us is,

that, adjust the microscope how one may, no evidence of internal structure is revealed in the clear transparent protoplasm of which the organism is apparently composed. It is true that, as regards differentiation in form, there is much to observe, *e.g.*, the ciliated gullet and the complex nuclear apparatus, but, as regards the ground substance which constitutes the mass of the *paramœcium*, beyond contained particles, no evidence can be obtained to warrant the ascription of any definite structure. The protoplasm appears as a clear, mobile, refractile substance. A very different appearance, however, presents itself at the onset of death, which is sharply differentiated from that to be observed in the living organism. A very fine network is seen to appear in the previously clear and homogeneous protoplasm of the organism and to impart to it a spongy structure. The spongy structure is at first only discernible with difficulty, but as death progresses it becomes coarser, until, apparently by a process of interfusion, it is easily detected with a comparatively low power of the microscope. This reticular structure of the cell protoplasm corresponds very closely with that observed by Bütschli and others, but with this important reservation, that I have been only able to detect it in the dead or dying organism.

Before proceeding to the consideration of the evidence that may exist of minute structures in the bacterial cell, it may be well to give a short *résumé* of the more important observations that have already appeared on this subject. Zacharias, working on the cyanophyceæ, was among the first to note and describe in oscillariæ, &c., a differentiation of the cell substance into cortical and central substances, and Bütschli, closely following and elaborating his work, comes to similar conclusions. Bütschli states that in such cells a differentiated central body can be detected, which he regards as the cell nucleus. This central body is readily stained with dyes such as hæmatoxylin, and contains still more deeply-staining granules. Similar granules are at times to be seen in the cortical layer which, together with the nucleus, is regarded by Bütschli as having a *reticular* structure ("wabigen bau"). The observations have been

extended to the bacteria with results of a similar character. The identity of the central body with the nucleus of higher plants is ably disputed by Fischer, who regards the appearance of a central body as a result of the contraction of the cell plasma, the contracted mass hanging by simple threads to the cell membrane. It will be readily understood that in this difficult field of observation, dealing as it does with the most minute forms of life, great differences of opinion continue to exist, not only with regard to the results obtained, but also as to their interpretation. Bütschli and Fischer may be taken as the most distinguished representatives of the opposing points of view. At the same time, careful work has been carried out with special reference to the bacterial cell by Babes, Hueppe, Migula, Schottelius, Trambusti and Galeotti, Zettnow and others.

It will hardly be possible to deal satisfactorily with the details of their work within the limits of the present paper. A very full account of the literature of the subject is given in Dr. Migula's *System der Bakterien*, Erster Band, 1897, and at the end of this paper references are given to the researches of greatest value.

Migula, after weighing carefully the various theories and observations, including those of Bütschli and Zacharias, is of opinion that the following may be accepted: The entire protoplasmic contents of a cyanophycean cell consists of an outer coloured and of an inner colourless part. The outer coloured part can be regarded as chromotaphor, although it is not yet quite certain whether it is separated from the cell membrane by a colourless layer of plasma. The inner central portion (Bütschli's "Central body") differentiates itself from the peripheral plasma layer, notably in staining properties. Whilst not a cell nucleus in the strict sense of the word, it may be regarded as a rudimentary form of the same. The granules that occur in the cell contents of the cyanophyceæ are probably of different nature, and it is not yet possible to decide their exact relations to the inner and outer layers of the cells containing them. The experiments made by Migula with typical bacteria to find if possible a "Central body" in Bütschli's sense were always negative, and he

concludes that such a body does not exist in the great majority of bacteria. It is, however, true that certain larger forms, such as beggiatoa, which have with doubtful propriety been classified with the bacteria, do contain Bütschli's "Central body." Migula finally suggests that the granules to be met with in bacterial cells may perhaps represent the first commencement of an intracellular nucleus.

The following pages are devoted to an account of the observations the writer has himself carried out. Any experiments in this direction make a large demand on the time and patience of the observer. Further, a careful education of the eye is necessary in order to detect the minute structural details in question, and more especially is this so for anyone accustomed to the ordinary conditions of bacteriological work with over-stained specimens and bright illumination of the field. The sensitiveness of the retina is thereby greatly impaired. It is only when working with a light screen and in a darkened room that the more delicate details of a cell begin to reveal themselves.

The objects of the present investigations were—(1) to devise a method of studying bacterial structure which could be employed with the certainty that such details of organisation as might be made evident by its means accurately represented the structure of the living organism; (2) to study the structures so made evident.

The organisms investigated were—B. typhi abdominalis, B. coli communis, B. mesentericus vulgatus, B. megaterium, B. proteus, B. subtilis, B. prodigiosus.

Apart from differences connected with the process of spore formation, the observations on these various forms resulted in such unanimity of appearance that it will be best to describe the net result as applicable to all the above forms. The process of spore formation is a distinct one, and the granules which take part in it are quite *distinct* both in appearance and reaction to those which will be described as playing a part in the life processes and structures of the non-sporing cell. It seemed a more satisfactory, though a more difficult, task to study structure in such organisms rather than to investigate the larger and more amenable

group of the cyanophyceæ. The relations of these forms to the true bacteria is by no means clear, and it would be in any case a dangerous assumption to correlate definite appearances observed in the larger forms with superficial similarities in the smaller types of cell.

After a large number of dyes had been investigated, roseine was finally selected as being the most useful for the purpose of this research. Methylene blue was also frequently employed. The usefulness of roseine is comprised under the following heads :—

(1) It is soluble in water, broth, and normal saline solution, and is a dye that can be used without causing the death of the organism. The whole of my observations have been carried out on the living cell, as I am strongly of opinion that the methods frequently employed with the resultant death of the cell do not yield results of a trustworthy or conclusive nature.

(2) Roseine possesses a distinctive and constant differential staining power for certain parts of the bacterial cell. How great this differential power is may be made evident by deeply staining an organism with a watery solution of methylene blue and then counter-staining with a very dilute solution of roseine. The blue staining is expelled from certain parts of the organism, which at once assume an intense red colour.

(3) Organisms stained with roseine, at least in the strengths used by me, are not killed. I have repeatedly satisfied myself on this point by inoculating a stained preparation on to agar or gelatin, when a copious growth always took place. Indeed, the organisms can be grown on media, liquid or solid, which are deeply stained with roseine. Under such circumstances they take up the stain as they grow, and may be thus conveniently examined in broth without further treatment.

No account was taken of any appearance seen in a preparation that was not unmistakably living at the time of examination, and no fixing or preservative re-agent was at any time employed. The stain was simply dissolved in broth and added to the culture that was under examination.

A hanging drop culture is not suitable for working with the restrictions imposed by oil immersion condensers and with the microscope in an inclined position, nor is such a method of preparation best adapted to the study of motile organisms. The method adopted was to simply place a minute drop of the stained culture on a slide and cover directly with a cover-slip. A drop of the culture was delivered from a capillary pipette on to the centre of a slide, and at intervals around it small portions of the stain were placed. Then by means of a platinum needle the drop of culture was mixed with the stain, and examined at once under the microscope, when, if not found satisfactory, more of the stain which was found to be insufficient was added, and the preparation examined again. In this way the relative quantities of the various staining reagents employed could be graduated with the greatest nicety, much in the same way as a painter mixes his colours on his palette. Using this method and with practice the amount of fluid under the cover-glass can be so graduated as to limit the movement of motile forms without crushing them or preventing limited excursions taking place. To prevent evaporation while the examination is in progress a film of vaseline or oil may be placed round the edge of the cover-glass. It is preferable, however, not to do this, as it renders the preparation too permanent, and, with the greatest care, changes leading to the disintegration of the organism will occur and erroneous results may thereby ensue. It is better to make a continuous series of fresh preparations and so avoid the risk of artificial appearances and products. It is quite certain that the above methods of investigation do not plasmolyse the organisms. It might be objected that some of the appearances about to be described are simply the result of such a process. But not only is plasmolysis rendered practically impossible by using only extremely dilute solutions of the dyes employed in the actual media in which the organisms have been growing, but, in addition, careful comparative experiments in which the cells were intentionally plasmolysed left no doubt that the appearances seen are not the result of such a process. The irregular grouping of the protoplasmic

masses in a plasmolysed cell is quite a different thing from the orderly arrangement and regular sequence of events to be witnessed in a normal cell. Further, in an intentionally plasmolysed cell the aggregated masses of protoplasm are changed in appearance by the salt employed. They become more refractile and denser in appearance. This appearance, when once seen, is easily recognised.

The errors of interpretation may be considerable in work of this character.

If a bacterial cell containing a large number of small discrete granules be examined with a narrow illuminating cone of light the appearance is that of a network or reticulated structure. The rings of false images surrounding each granule overlap each other, and also similar rings from granules on a different plane. The intersection of these images gives rise to the appearance of a definite network. If, however, the angle of the illuminating cone be increased these false images vanish, and each granule stands out discrete. It is important, therefore, in all cases where minute structure is being investigated, to employ as large a cone as possible, so as to avoid manifest errors of interpretation. This ideal method has, however, its disadvantages. Chief amongst them is the difficulty of getting, with the flood of light a wide cone necessitates, sufficient contrast for clear vision. This difficulty may, however, be very largely overcome by using light from the violet end of the spectrum only. If the light from the lamp before entering the condenser system be passed through a Gifford's screen, a great improvement in the image is obtained. Such a screen is constructed by dissolving methyl green in hot glycerine, placing the solution in a glass tank and immersing in the liquid a piece of Chance's signal green glass. If constructed with care the red end of the spectrum may be cut off to the line F.

The use of this screen has another great advantage, viz., that by its use even completely unstained organisms may be examined, and with comparatively wide illuminating cones; a method that is almost impossible without its use.

If a young and vigorous culture of any of the micro-

organisms enumerated above as having been the objects of investigation be examined by the aid of the simple method just described, cells in all stages of growth will be found. It becomes necessary, therefore, at the outset to determine which of the many appearances seen is to be taken as the *standard* of comparison. I propose to take as such a standard a cell which is about to divide, or has just divided. The reasons for this choice are, that not only does such a cell give evidence of its activity by the fact of division, but, in addition, when a cell is dividing its structure will be found not to be complicated by such additional processes as spore formation or involution phenomena. Such a cell may be called an *actively living cell.* Its appearance is as follows, and the results described are fairly constant. The bulk of the cell will either appear unstained by roseine alone, or if the double staining method with methylene blue and roseine be employed, the mass of the protoplasm will be stained blue. Conspicuous objects in both methods are small spherical refractile granules, from one to three in each cell, which are coloured a vivid red. The brilliancy of the colouring of these granules is remarkable, considering the extremely dilute solution of the dye employed. The cell is turgid and full of the blue coloured material when doubly stained. This material is absolutely structureless, the most that can be said for it is that it is very finely granular. The most careful adjustments with the best microscopical appliances now available quite failed to show any sign of structure in the blue stained portions of the cells. Indeed, the homogeneous nature of the cell plasma can be directly deduced from the often repeated observation of watching a cell containing granules roll over and over across the field of the microscope, when the various contained granules will be seen to eclipse each other according to their position only, *i.e.*, they are always visible as refractile particles through any portion of the transparent cell plasma and only become obscured when two come into the same line of sight. Did anything in the nature of a honeycomb or reticular structure exist, the appearance would be very different. Under these circumstances a granule, seen

through a layer of anisotropic plasma, would vary in appearance with the thickness of the intervening material, which is, however, certainly not the case. No suggestion of any cell wall is obtainable at this early stage in the life of the cell without the employment of artificial methods such as plasmolysis. In this, the most active stage of its life, the bacterial cell consists, as far as can be ascertained with legitimate methods, *i.e.*, excluding plasmolysis, and with the existing lenses, of a minute mass of cell plasma containing one or more granules. Such a cell may be either the result of a just completed cell division or the first cell that has originated from a spore. In this condition it can divide and give rise to another cell of precisely the same kind, or it can give rise (in spore-forming species) to a spore, or it can proceed to the notable series of changes about to be described. The cell in the above stage is represented, drawn as accurately as possible from an actual living preparation, in figs. 14—20.

If the actively living cell proceeds to divide it will increase (1) in length, and (2) the number of the granules will increase.

The increase in length occurs simply by a process of growth; the increase in the number of granules takes place in two ways. New granules appear in parts of the cell previously destitute of them, and the already existing granules, having increased in size, divide into two. When the cell is of the necessary length for division, this takes place by constriction. The constriction in the majority of cases passes through and involves one of the granules which was situated in its path (figs. 21 and 22). Two cells now occupy the position of the parent cell, and each is precisely similar to it and to the other and each contains granules. This process may be repeated indefinitely, and as long as it takes place no other structure than that described can be observed. If, however, the cell proceed to spore formation, the condition of simplicity is lost. Such a cell, at first indistinguishable from the actively living cell, soon bears evidences of its impending fate in the development of refractile, oily-looking droplets

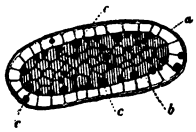
not staining with roseine. These droplets run together and ultimately give rise to the spore. While this is going on changes are taking place in both the cell contents and in the granules. As the oily substance increases in quantity the cell plasma diminishes, at the same time receding from the periphery and leaving a clear space between itself and what now becomes evident as a cell wall. The granules increase in number and aggregate into two spherical masses, which dispose themselves symmetrically at each end of the cell with the now large spherical spore material interposed between them. Round the spore is the shrunken plasma (*v.* fig. 24). The cell membrane now becomes very evident and, collapsing on the contents, follows the contour produced by their presence. The further fate of this composite structure lends additional support to the actual entity of the cell membrane. When the spore is fully formed the cell-case breaks across the line a—b, allowing the spore to escape and leaving two cup-shaped receptacles, in which may still be recognised the granules referred to and a minute remnant of the now effete cell plasma (fig. 25).

As to the process which leads to the formation of the *spore membrane*, very little can be said. Presumably it is secreted by the residual layer of protoplasm which aggregates round the forming spore. The material which collects in droplets and which ultimately runs together to form the spore is quite structureless to present methods. It is more hyaline and more refractile than the plasma of the actively living cell, and resembles in appearance the material produced from the cell plasma when a bacterial cell is plasmolysed by concentrated salt solutions.

The actively living cell may thus either divide or proceed to spore formation, but still another fate is open to it. What the ultimate result of this process may be I have not yet been able to ascertain, but from the regulated order of its successive stages it would seem probable that an important event in the life history of the organism is subserved by it. The process, which we may call the process of granule formation, is as follows :—

The cell which is not going to divide or to sporulate first gives evidence of the impending process *by the more rapid multiplication of its granules.* These increase in number both by division and new formation, and become arranged in a fairly regular layer around the periphery of the cell (*v.* fig. 14). No other change is observable at this stage in the configuration of the cell plasma. Signs of alteration, however, soon become manifest. By some process of condensation it commences to aggregate centripetally. A narrow zone or clear space becomes evident immediately subjacent to the cell membrane. The cell now presents the appearance of figs. 7 and 8.

The shrunken cell plasma preserves a hyaline appearance, and scattered through it are a number of granules. This stage viewed with a narrow cone of light is very suggestive of Bütschli's diagrammatic representation of the



normal structure of a bacterial cell, which is reproduced in the accompanying woodcut; but with a wide illuminating cone the reticulated appearance vanishes, and each granule which appeared at the nodal points of the network is seen to stand out clear and distinct by itself, and all appearance of a web vanishes. If such a cell be double stained, the centrally contracted plasma stains blue and the granules red. The space between the central body and the cell wall remains unstained, while the *case* itself takes on a delicate pink colouration.

This stage is, however, but preparatory to the next. The central shrinking continues, and the granules become less numerous. The case remains distended, and preserves to the end the original configuration of the actively living organism. The shrinking of the central mass continues, and the diminution in the number of the granules keeps pace with it until a stage is reached which is represented in figs. 9, 10, 13.

Here the cell plasma has contracted to a centrally placed body suspended in the midst of the cell in a fluid substance. And now commences a remarkable phenomenon.

The central mass, composed of the remnants of the cell plasma, enclosing from three to six granules, commences to move. This movement, at first a mere tremor, rapidly gathers force until, when at its maximum, it presents a combination of movements of translation, vibration and revolution. How long it lasts I am unable to say, but I have watched an individual specimen in this stage for nine hours without observing any alteration in the character or diminution in the activity of the motion. The fact that such a movement is possible demonstrates that the contents of the cell at this period are fluid. That the consistency of the fluid need not be less than that of, say, cedar wood oil is shown by the following experiment: If some egg albumen be shaken up violently in a tube with cedar oil and finely divided colouring matter, such as carmine, a fine emulsion is formed in which many of the individual particles are less than 5 μ in diameter. If search be made amongst such particles some will be found in which a hollow sphere of albumen encloses a droplet of oil in which are suspended particles of carmine. If these particles be watched they will be found to exhibit a movement in many respects similar to that described above. It differs from it perhaps in the movement of translation being of less extent. This observation also indicates that the movement may be due to purely physical causes of the same nature as those which cause Brownian movement or pedesis.

Occasionally isolated granules are observed to participate in the movement at this stage, that is, granules that are not held together by the contracted cell plasma. What the end of this process of plasma contraction is I have been unable to ascertain; cultures in which this stage was frequent have been kept for months without showing any further change.

The effect of these observations is to confirm the belief that a bacterial cell consists of the three elements: (1) cell wall, (2) cell plasma, and (3) granules; and to limit the conception to such structures. It will be convenient to

consider further these several structures and to study them individually in the light of previous work as well as of my own.

1.—*The Cell Wall.*

The existence of a cell wall to the bacterial cell was postulated as long ago as 1875 by Cohn. Cohn's reasons, however, for assuming the existence of such a structure were purely theoretical, and it was not until Fischer objectively proved its existence by causing the cell plasma to shrink away from its enclosing case that any solid reasons existed for regarding it as a demonstrable structure.

Ingenious as Fischer's method was, it labours under the stigma of being an artificial method, that is to say, an appearance that could be interpreted as a cell wall was only observable after a violent physical change in the structure of the organism. Its actual existence can, however, be proved in the living cell. In stained preparations of living individuals in the stage of granule formation, when the cell plasma has shrunk round the centrally placed granules, the cell membrane can in nearly all cases be seen of a delicate pink colour, and in those cases in which it can not be seen its existence can be inferred from the limitation of the movement of the centrally placed mass, especially the limitation of the movement of translation, to a space corresponding in extent to that occupied by an actively living cell. By staining too with a slightly acid aqueous solution of soluble blue the cell membrane takes on a blue tinge and can be recognised as a distinct structure.

Again, amongst the later stages of the process of spore formation individuals are found from which the spore has just escaped, leaving the cell wall as two cup-shaped structures, the rim of the cups being the line of rupture of the case (fig. 25). In these stages the cell membrane is a rigid structure, as is evidenced by the retention of the cup-shaped form in the example last mentioned, but it is not clear that in the actively living cell the cell membrane is of the same nature. For, as has been observed by myself, the granules may be seen at times to migrate through the cell wall; in

such stages, therefore, we must assume an elastic consistency of the cell wall (figs. 23, 16, 21). It would seem, therefore, that the cell wall is a structure of gradual formation—existing in the earlier stages—in the actively living cell, as a delicate elastic envelope and gradually ageing into the condition of a rigid and brittle membrane. As to the relation of flagella to cell wall, I am not able to speak as yet. The observation of flagella in living specimens is a matter of extreme difficulty; possibly the first-mentioned progressive increase in rigidity of the cell wall may have an important bearing on the question, especially if found to be associated, as I have reason to suppose, with the final loss of these organs.

2.—*The Cell Granules.*

The most striking feature of the cell granules is their intense colouration with roseine. An aqueous solution of this dye, so dilute as to be only just pink, will stain the granules a brilliant red. As to the part they subserve in the life of the cell, what little that can be added to the already described observed phenomena is purely speculative and theoretical. The first obvious suggestion is that they are nuclear in nature. The idea of a substance within the cell intensely reactive to staining re-agents, controlling and directing the functions of the cell, is now such a common one that instinctively any deeply staining mass within a cell is at once pronounced as nuclear in nature. The fact already mentioned, that a granule can frequently be seen to divide with the cell, seems at first sight to lend strong support to such a view. But their apparently indiscriminate distribution, and the fact that they are not always equally numerous in the products of a cell division, seems to show that all of them at least are not nuclear or directive in nature. In some specimens, again, in the early stages, *i.e.*, stages not far removed from the actively living condition, they may be seen to become extruded from the cell and to exist freely in the surrounding medium, which is not in accordance with the usual conception of a nucleus. It is true that in the extrusion of the polar globules after

fertilisation, or preparatory to it, in the case of the male element in animals, we have a similar phenomenon in structures undoubtedly nuclear, but this occurs as part of a very specialised process, and, as far as is known, as part of that process only.

It seems more likely that the granules may be regarded partly as excretory products in the case of bacteria, and the fact that the cell plasma surrounding them is different in appearance from the bulk of the plasma of the cell would lend support to such a view. Around each granule may always be made out a clear space which gradually fades into the less refractile cell plasma. Such a space indicates a differentiation in the cell substance which might very well be correlated with more active excretory processes. On this view the granules would be the contents of intracellular secretory structures, much in the same way as the eosinophil granules of a white blood capsule have been so regarded. But if they are to be regarded purely as excretory mechanism—and the fact of their spontaneous extrusion would support such a conception—why is it that they so often take part in the division of the cell, as I have observed. To reconcile the two conceptions, it must be admitted either that the granules are not all of the same kind, some being, perhaps, nuclear, and some excretory, or that a nuclear structure is excretory in nature. On the latter view, in order to admit the necessity of a nucleus dividing with the cell, it may be supposed that such necessity is a consequence of a limited amount of protoplasm being able to accommodate only a certain amount of effete material; on the reduction of such quantity of protoplasm by one half at the moment of division, it becomes either necessary to share the stock of such material equally between the resulting cells, or to extrude it, which, as we have seen, is frequently done. But discussion such as this without supporting facts is not of much value.

Bütschli has suggested that the entire mass of the bacterial cell is nuclear in nature. This statement he bases on the great affinity of bacteria for staining reagents, a property conspicuously attributable to nuclei in general.

But it may be doubted whether bacteria mass for mass of stain have such affinity. The strength of the solutions of aniline dyes usually employed is out of all proportion to the actual amount of bacterial substance present even in the thickest preparations, and if, as I have stated, dilute solutions be employed, a very marked differentiation in staining activity is observable.

3.—*Cell Plasma.*

As regards the cell plasma I have not much to say, beyond the fact that it appears structureless when legitimate methods, not involving the death of the cell, are employed.

The process of spore development especially in relation to the granules is deserving of notice.

The spore having been formed as already described, if it is placed in favourable conditions it will germinate. This may be observed most conveniently on the hot stage of the microscope, the spores being placed in broth to which a faint red tinge has been given by roseine. The process to be described was observed in this way on the spores of B. mesentericus vulgatus. Such a spore is invested in a very thick spore case, and the first process observed is the rupture of this case. Until this takes place the spores remain quite colourless, the dilute solution of roseine employed being quite insufficient to penetrate the thick spore membrane. The rupture of the case takes place in many different ways, some of the more curious of which are shown in figs. 1 to 6.

In fig. 1 the case is seen rolling back over the contained embryo; in fig. 4 a simple median splitting has taken place, the resulting caps being separated by the growth of the embryo; in fig. 6 a longitudinal splitting has occurred, one half of which is rolling back, much as in fig. 1. Many other varieties of method of removal of the case have been witnessed, but all result in the common end of setting free the contained embryo. This at first is spherical in shape and highly refractile in appearance. It soon, however, loses its transparency, especially at one pole, that at which the first cell grows out. It always contains a conspicuous granule deeply

stained red if examined by the method described. The further changes in the embryo are slight. The refractility vanishes and very soon an actively living cell is formed. This appears to grow out from one pole of the embryo, which, in consequence, becomes flask-shaped, the more acute end being that which is actively growing, the more rounded end being the remains of the embryo now rapidly losing its refractile appearance. The granule which is always present in the embryo at the moment of emergence from the spore case apparently splits into two, one of which takes up a position at the growing point. A curious structure is noticed in figs. 1 and 2 on the side of the emerging embryo. It consists of two minute projections. This is very constantly present, but I am unable to make any suggestion as to its nature or function.

The conclusions I have been able to reach up to the present are as follows:—

(1) In the actively living bacterial cell, or one which is about to divide, when examined by methods which do not cause its death, *no* reticular structure can be demonstrated.

(2) An organism in which structure is visible is not an actively living cell, but is progressing either to spore formation or to granule formation.

(3) In an actively living bacterial cell which consists of cell wall, cell plasma and granules, the cell wall is a progressive formation and becomes finally a rigid structure.

(4) The cell plasma is hyaline or very finely granular. The granules are refractile and spherical and stain vividly with roseine.

(5) The granules may participate in cell division.

(6) The granules may be extruded from the cell through the cell wall.

(7) The embryo on emergence from the spore case contains a granule, the presence of which in such an early stage lends support to the view that the granules are fundamental elements of the bacterial cell.

The investigation of the flagellated and capsulated forms of bacteria is at present in progress, and in a subsequent paper reference will be made to these structures as well as

to the observations that are being continued on the above lines. I have to thank Dr. Wunderlich for the aid he has rendered in the earlier and more difficult stages of this research, and I am indebted to Dr. Macfadyen, without whose help and advice this work would not have been completed.

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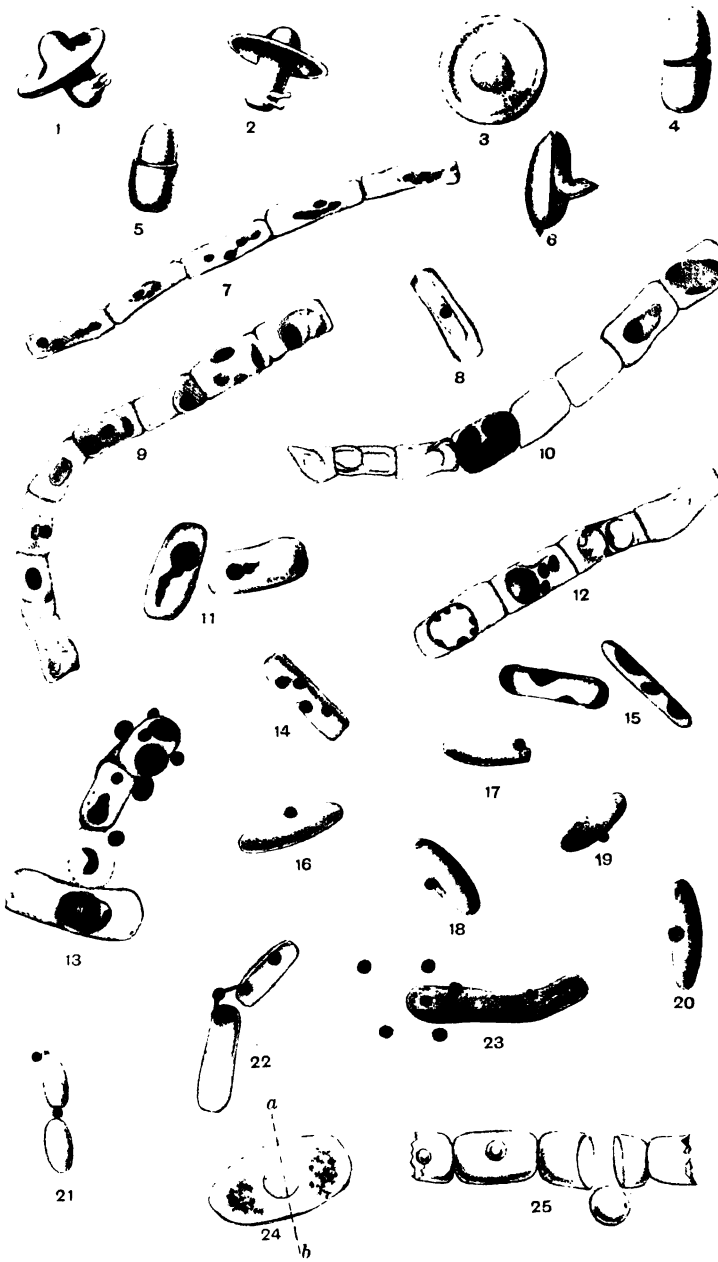

OBSERVATIONS UPON THE STRUCTURE OF BACTERIA.

Bale & Danielsson, Chromo-Lith.

DESCRIPTION OF FIGURES.

Embryology of the Spore.

Fig. 1 to 6.—Stages in the embryology of Mesentericus vulgatus showing the various methods in which the spore membrane is split.

Fig. 1, 2 and 3.—Different views of the symmetrical unrolling of the spore membrane. In figs. 1 and 2 the appendages referred to in the text are shown.

Fig. 4 and 5.—Two stages in the method of equatorial splitting of the spore membrane.

Fig. 6.—Another method of splitting of the spore membrane.

Fig. 15.—The actively living cell of Proteus vulgaris showing massive grouping of the granules.

Figs. 16 to 20.—The actively living cell of B. prodigiosus. In figs. 18 and 19 the granules are shown leaving the cell through the as yet ill defined cell wall.

Fig. 21.—Division through a granule together with extrusion of the granules.

Fig. 22.—Another stage of division through a granule which was frequently observed. A connecting strand of granule substance with a mass of the same material is well shown.

Fig. 23.—A cell of B. prodigiosus about to divide and at the same time extruding its granules. The division will take place at that part of the cell where the two granules are seen.

The Process of Granule Formation.

Fig. 14. The first stage in the process showing multiplication of the granules and commencement of centripetal shrinking of the cell plasma.

Figs. 7 and 8.—Subsequent stages. Further shrinking of the cell plasm with a well defined clear space (containing fluid) between the central mass and the now well defined cell wall.

Figs. 10, 11, 12.—Various appearances; the later stages of the process of granule formation.

The Process of Spore Formation.

Fig. 24.—An early stage in the process. The refractile granules have run together into a central mass. At each end of the cell can be seen the aggregations of the roseine stained granules.

Fig. 26.—The completely developed spore emerging from the now empty cell case. Note the preservation of the form of the empty case, indicating a rigid structure.

THERMOPHILIC BACTERIA.

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(*Second Paper.*)

THE first observations we made upon bacteria growing at high temperatures were published in the *Journal of Pathology and Bacteriology* in 1894.[1] These observations showed that such organisms were of great variety and widely distributed. They did not occur as isolated examples, but constituted a group to which the term of thermophilic bacteria might be applied.

At that time the subject of thermophilic bacteria had been but little investigated—beyond the isolated observation on the bacillus thermophilus by Miquel, and the partial study of some similar forms by Globig.

Some months after the appearance of our paper a research on similar lines was published by L. Rabinowitsch.[2] This paper, which will be referred to subsequently, contained practically a confirmation of our results, as well as to a certain extent a repetition of the theories we had brought forward regarding the possible conditions for the growth of such organisms. There have also been a few other communications published, and these will be referred to in the course of the paper. At the same time, there has been no prolonged investigation of the group as a whole. The subject appeared of such interest, as well as the problems connected with it, that we continued its investigation. The following results have confirmed our previous investigations, and have also brought out some new facts. The experiments extended over a considerable period of time (two years), and were

made upon a large amount of material, so that there might be adequate data for any conclusions reached. The details also are fuller with regard to the cultural and other peculiarities of the organisms. In our previous paper the conclusion we had reached was that there is a widely distributed group of organisms in nature which find their optimum temperature of growth between 55° and 65° C. At that time we isolated from various sources and described fifteen forms which not only grow best at these high temperatures, but also retain all the activities of the living cell. The further details will be found in the paper cited.

The wide distribution of these organisms is of a very striking nature. The material examined by us was collected from the most varied sources and cultivations made at 55° to 65° C.

The organisms were found in Thames water, Thames mud, sewage water; and in sea water from Plymouth a thermophilic bacillus was detected. They were also detected in the surface and deep layers of the soil, and in dust from the streets and house roofs, as well as in the intestine of man and the horse. They were present in the digestive tract of ducks, fowls, mice, rabbits, guinea-pigs, worms, frogs, and snails. Manure heaps, as one would expect, contained large numbers. Amongst fish they were found in the digestive tract of goldfish, carp, eel, and whiting, as well as in oysters. They were present in straw and in all samples of ensilage examined. They were not detected in tap water or in sputum. The results were also negative in the case of a thermal spring at Bath (T. 118° F.). A sample of volcanic dust from Japan, accidentally obtained, gave no growth.

Amongst other experiments a freshly dead mouse was placed with a little water in a sealed jar, and kept at 65° C. The mouse became mummified, but no putrefactive decomposition occurred. The ordinary putrefactive bacteria were killed by the heat, and the thermophilic organisms appeared incapable of producing a decomposition of the animal. Further, the ordinary bacteria of putrefaction were not capable of adapting themselves to a thermophilic temperature. They are excluded from any chance of being mixed up with the

thermophilic bacteria. A large number of organisms were isolated, for which the optimum temperature of growth is 55° to 65° C. They possess this peculiarity as their cardinal feature. The existence of these organisms may be demonstrated in the following simple manner :—A sample, say of soil, is placed on sloping agar, and incubated at 65° C. A rapid development occurs, and in fifteen to seventeen hours the whole agar surface is covered with a growth. From this growth agar plates of various dilutions are made. Colonies of differing character will appear, indicating the presence of more than one kind of thermophilic organism.

Thus we may observe an anthrax-like colony, a colony resembling B. figurans and smaller round whitish colonies on the same plate. The plates are frequently entirely covered with a thin filmy growth, which microscopically appears to be a pure culture of one organism. This frequently prevents one obtaining subcultures of deep colonies in the agar without contamination by the surface growth. A larger series of plate cultures did not always overcome this difficulty, and the subcultures were frequently found to contain mixed growths. This tendency to diffuse surface growth of one species proved troublesome, and had to be checked. The addition of various substances to the nutrient agar was tried with this end in view, *e.g.*, 2 per cent. sodic chloride, glycerine, &c. The results, however, were not satisfactory. After some experimenting we obtained in potato agar a soil which gave good results. The organisms grew on this soil as isolated colonies, and remained discrete, and it was possible to obtain readily pure subcultures of the organisms. This soil proving the best, all the subsequent isolation experiments were carried out with it. One need not fear a drying up of the nutrient medium at these high temperatures, as the growths that occur are so rapid. A large number of thermophilic colonies were in this way obtained from soil, &c.

The potato agar is prepared as follows :—Potatoes are first steamed, peeled, and pounded. To 100 grammes of potato is added one litre of tap water, and the mass is steamed for half an hour and filtered. To the filtrate 1·5 to

2 per cent. of agar is added, and the whole autoclaved for fifteen minutes. It was found an advantage to add 1 per cent. of salt. After neutralisation with soda and further steaming, the potato agar is filtered into test-tubes and sterilised once more. It will be seen that this is practically a carbo-hydrate soil; it will be referred to as salt potato agar.

From the primary colonies obtained on this soil subcultures were made on the ordinary culture media, on which as a rule thermophilic bacteria readily grow. The organisms can also be readily cultivated from generation to generation on this soil at high temperatures—a further proof of their thermophilic character.

As regards cultural peculiarities, we tested other soils, such as slices of carrot, beet, and turnip, with unfavourable results. In many instances no growth occurred, or it was of a diffuse character. Naegeli's nutrient salt solution did not furnish any perceptible growth, but growths appeared upon the addition of a trace of peptone broth. Globig, and also Rabinowitsch, used the potato for isolation purposes. We did not, after testing the point, select potato for this purpose, because the growth of the organisms is often slow; some will not grow on it, and the colonies that do occur are frequently so mixed that it involves much trouble to separate pure cultures.

Notwithstanding frequent attempts, several of the organisms could not be made to grow upon simple potato. The potato, though used by the above observers, is not a suitable soil for isolating all the varied forms of these bacteria that undoubtedly exist. Those that do grow on potato generally give characteristically pigmented growths, easily differentiated by the pigment produced, and especially the forms occurring in soil. For example, a sample of soil sown directly on potato gave the following pigmented growths:—Canary yellow, white crusty, umber, cream, dull brick, buff, and brownish coloured growths. It is interesting to find such bright pigment production occurring at 56° C., and it is another instance of the full vitality of the organisms at this temperature.

After these preliminary experiments we selected salt potato agar for isolation purposes instead of the ordinary

agar originally employed. From the colonies and their sub-cultures microscopical specimens were made, and showed without exception bacillary forms. At times long thread-like and interlacing filaments were visible, but we were unable in any instance to demonstrate genuine branching forms. Their growth was also studied on gelatine, serum, potato, milk, &c., and in this way many useful differentiations were brought out. It became speedily apparent that we could not hope to describe exhaustively all the forms that presented themselves, and a process of selection had accordingly to be adopted. We selected frequently recurring organisms as characteristic and typical of the group, and their morphology and biology were more closely studied than had been done in our previous paper. It is proposed to give a description of fourteen forms, and some illustrations are given of the most characteristic colonies. It has not been thought advisable to give them names, but to simply distinguish the organisms as Bacillus I., II., III., &c.

The various organisms stain with the ordinary dyes, but, on the whole, carbol. methylene blue was found to give as good results as any. It was usually found advisable, before staining, to treat the cover-slip specimen with dilute acetic acid. This cleared away a zooglœa-like membrane, which often interfered with clear staining, and such specimens gave better results.

The following is a description of the colonies as they appeared on salt potato agar, along with the microscopical peculiarities, &c. The subsequent table gives the main cultural appearances of the growths obtained, indol reaction, &c.

Morphology, &c., of the Thermophilic Bacteria.

In all instances the description of the colonies refers to a twenty-hours' growth on salt potato agar at 55°-60° C. The hanging drops were made from six-hours' cultures, to avoid sporing forms :—

Bacillus I. (from animal dejecta).—Marked surface growth, colonies 8 to 10 mm. in diameter, yellowish white, resemble small ball of cotton-wool with teased out margin. × 50,

appear yellowish brown, anthrax-like, tendril-like runners on agar surface, forming loops and convolutions (fig. 1).

Organism non-motile, rods 3 to 4 times as long as broad; average, L. 3 to 4 μ, Br. 1 μ. Form long chains, frequently apposed and parallel; also looped and twisted with pseudo-branching arrangement; ends rounded, in chains frequently flattened.

Gram:—Negative; spores towards centre of slightly swollen rod.

Bacillus II. (from animal dejecta).—Surface colonies about 2 mm. in diameter, dull white, round, smooth contour. × 50, appear yellowish brown, finely and uniformly granular, slightly wavy at margin (fig. 2).

Organism non-motile, rounded ends; L. 4 to 6 μ, Br. 1·25 to 1·5 μ.

Gram:—Positive, oval spore towards one end of rod, somewhat larger than bacillus, and giving a "fishfloat" appearance.

Bacillus III. (from soil).—Small pinhead colonies, dull whitish colour. × 50, appear yellowish brown, finely fringed margin, finely granular with delicate wavy linear markings running towards periphery.

Organism non-motile, large rods, about five times longer than broad, ends rounded, tendency to form short wavy chains; no false dichotomy; L. 6 to 8 μ, Br. 1 μ.

Gram:—Positive; large, spoon-shaped terminal spore.

Bacillus IV. (from ensilage).—Dull white, spherical colonies, about 1 μ in diameter, with mottled appearance. × 50, appear round, pale yellow, coarsely granular, and smooth contour (fig. 3).

Organisms present oscillatory movements, otherwise non-motile; a short rod; L. 3 to 4 μ, Br. 1 μ, ends rounded, single and in pairs; no chains.

Gram:—Positive; small oval spore towards slightly swollen end, and occupying about three-fourths of bacillus.

Bacillus V. (from Thames mud).—Spherical, yellowish white colonies with regular margin, about 4 mm. in diameter. × 50, appear dark brown and finely granular in central zone, which occupies three-fourths of colony; outer zone is pale

yellow, coarsely granular, with ridged markings, and slightly teased out at margin (fig. 4).

Organisms have oscillatory motion; otherwise non-motile; resembles Bacillus IV., but more slender, ends rounded, short wavy chains.

Gram:—Negative; spores freely, spore terminal and about same diameter as rod.

Bacillus VI. (from Plymouth seawater).—Dull white, and feathery colonies. × 50, are characteristic; irregular shape and of a fleece-like texture throughout; somewhat resemble finely teased out cotton-wool fibres in the agar (fig. 5).

Organisms non-motile, large slender rods, tendency to pointed ends and chain formation; L. 6 to 10 μ, Br. 1 μ.

Gram:—Negative; drumstick spore, giving "bonnet-pin" appearance.

Bacillus VII. (from soil).—Round, dry, feathery colonies, dull white enamel appearance, 4 to 12 mm. in diameter. × 50, appear brown and feathery, central markings, finely granular at margin, which likewise shows fluted markings and indentations (fig. 6).

Organism non-motile, rods about size of anthrax bacillus, ends slightly flattened, in pairs and short chains.

Gram:—Positive; large, spoon-shaped terminal spore.

Bacillus VIII. (from soil).—Yellowish white, spherical colonies. × 50, appear yellowish brown, "biscuit" appearance, distinct rimmed margin, contour finely serrated, colony is coarsely granular and shows three zones, an outer clear, a middle dark brown and a central light brown zone (fig. 7).

Organism, oscillatory, otherwise non-motile; rounded ends, and short curving chains; L. 4 to 8 μ, Br. 1 μ.

Gram:—Positive; oval central spore filling greater part of bacillus.

Bacillus IX. (from soil).—Marked, surface growth; spherical colonies, 4 to 12 mm. in diameter, dull ground glass appearance. Colonies ultimately assume crusted white appearance and show fine radial markings. × 50, present a characteristic frosted glass appearance, coarsely granular in centre with clear marginal zone (fig. 8).

Organism, non-motile, straight and curved rods, rounded ends, tendency to long thread formation; L. 4 to 8 μ, Br. 1 μ.

Gram:—Positive; very small and terminal oval spore.

Bacillus X. (from soil).—Dull white, circular colonies, about 2 to 8 m. in diameter. × 50, are characteristic, yellowish colour, and show two zones—an inner finely granular about three-fourths of colony, and an outer zone with deeply indented margin showing delicate lace-like appearance and fluting on surface (fig. 9).

Organism, motile, numerous scattered rods, occasional chains, ends rounded; L. 6 to 10 μ, Br. 1·5 to 2 μ.

Gram:—Positive; large terminal and oval spore.

Bacillus XI. (from soil).—Round, dull white colonies with moist, glistening appearance. × 50, appear yellowish brown, spherical, smooth contour, contents finely granular and freckled with faint markings, which, by reflected light, appear as a delicate reticulum.

Organism, non-motile, rods form long anthrax-like chains, straight and curved, ends rounded, about size of Hay bacillus.

Gram:—Positive; terminal and oval spore.

Bacillus XII. (from soil).—Stellate, yellowish white colonies. × 50, appear yellowish white, and margin somewhat resembles an anthrax colony on agar at blood heat (fig. 10).

Organism, non-motile, forms long chains, ends somewhat flattened; L. 4 to 8 μ, Br. 1 to 2 μ.

Gram:—Negative; a drum-stick spore.

Bacillus XIII. (from soil).—Round, yellowish white colonies. × 50, appear spherical, brownish, finely granular with smooth contour, not very characteristic (fig. 11).

Organism, non-motile, rods plump, square ends, form chains; L. 4 to 8 μ, Br. 1·5 to 2 μ.

Gram:—Positive; spore towards swollen end of bacillus.

Bacillus XIV. (from soil).—Thin surface film over agar, discrete and circular colonies at margin. × 50, appear granular, smooth contour, somewhat resemble a ball of cotton wool (fig. 12).

Organism, motile, short slender rods with rounded ends, surrounded by a zooglœa, no chains; L. 4 μ, Br. 1 μ.

Gram:—Positive; oval spore towards centre of rod, giving a "fish-float" appearance.

The following table (I.) gives the appearances of the growths of the organisms in the ordinary culture soils of the laboratory, and is a further indication of the existence of a number of individual species of thermophilic bacteria.

The following are the main results of the above experiments:—There is in the first instance the good growth of all the above forms at 55°-65° C. on the ordinary culture media. It may be mentioned that their growth was likewise tested in sterile urine at thermophilic temperatures. In two instances no growth occurred in this soil. Of the others, a few gave a cloudy growth with general turbidity of the urine, whilst in other instances the urine remained clear, the growth appearing as a flocculent deposit in the test tube.

The organisms were all sporing forms of bacilli, and we did not succeed in isolating other forms of micro-organisms. Dr. Kidzior[6] has, however, described a thermophilic cladothrix as present in sewage and in the river Spree. This organism grew within sixteen hours in broth at 60° C. and showed genuine branching filaments. The organism is facultative anaerobic, and its temperature optimum is 55° C. It appears to produce a resistant spore or "dauerform," from which the threads develop at suitable temperatures. The observations of Rabinowitsch and others agree with our own as regards the isolation of exclusively bacillary forms of a sporing character.

The colonies presented distinctive appearances, especially those developing on salt potato agar. The growths in most instances were remarkably rapid; in one case a growth occurred over the whole surface of the agar within *six* hours. As a rule, good colonies were obtained after incubation for one night at thermophilic temperatures.

The majority of the bacilli showed a tendency to grow in long chains, and whilst branching-like forms appeared, we did not detect any genuine dichotomy.

Two organisms from soil (Bacillus X. and Bacillus XIV.) were the only bacilli that possessed undoubted motility

when observed in young cultures. Gram staining gave in some instances positive, in others negative results. It will be seen from the table that a number of these organisms do not grow on potato; in the positive cases the pigment production was striking and characteristic. The great majority curdled milk, and in seven instances liquefaction of the gelatine was produced. The indol reaction was positive in most instances, and only in one instance was it obtained without the previous addition of a nitrite to the broth. The growths on ordinary agar were not very characteristic.

The ubiquitous character of these organisms has been confirmed by other observers. Eight species are described by Rabinowitsch[2] as occurring in soil, water, the large and small intestine, &c., and in one instance in germinating barley at 62° C. The spores resist steaming for five to six hours. Dr. Oprescu[7] likewise describes five forms of thermophilic bacteria, which he classifies as follows:—(1) bacillus thermophilus aerobius (sewage water); (2) bacillus thermophilus aquatilis (Spree water); (3) bacillus thermophilus liquefaciens aerobius (soil); (4) bacillus thermophilus reducens (unsterile serum); (5) bacillus thermophilus liquefaciens tyrogenus (Roquefort cheese). A few isolated observations are likewise recorded by others.

Temperature Conditions.

We now proceed to a series of experiments made with the view of determining the range of temperature for the growth of thermophilic organisms. The experiments were made with pure cultures on agar, plain potato and potato agar. The results are given in the accompanying table (II.) The series at 22° C. are not included, as in each instance no growth was obtained at this temperature. It may be said that the growths were slower at the lower temperatures, occurring after one, two and three days, and were quicker at the higher temperatures, occurring usually within twenty-four hours. The range of temperature for each organism will be seen by running the eye across the table, which also

TABLE I.

The Growth of Thermophilic Bacteria in Culture Media.

No. of Bacillus.	Source.	Gelatin.	Agar.	Broth.	Potato.	Milk.
Bacillus I.	Animal dejecta	Copious flocculent deposit. Liquefaction, 14th day	Diffuse growth; not characteristic	Ropy viscous deposit; supernatant broth clear. Indol reaction negative	No growth	No action.
,, II.	Animal dejecta	Flocculent growth throughout gelatin; marked surface pellicle	Diffuse delicate surface film	Delicate surface pellicle and deposit; broth otherwise clear. Indol reaction positive	No growth	Viscosity, but no separation of casein.
,, III.	Soil	Ropy growth; no pellicle. Liquefaction, 5th day	Discrete, moist yellowish-white colonies, with smooth margins	Distinct stringy pellicle and viscous deposit. Indol reaction positive	Cream-coloured growth, of brownish tinge in old cultures	Solid curd, no whey.
,, IV.	Ensilage	Copious flocculent deposit; no pellicle	Dull white slimy growth over whole surface	General turbidity; no pellicle. Indol reaction positive	No growth	Solid curd, no whey.
,, V.	Thames mud	General turbidity and crusty pellicle	Diffuse dry film	General turbidity; surface pellicle. Indol reaction positive	No growth	Solid curd, no whey.
,, VI.	Plymouth sea water	Practically no growth	Diffuse transparent film	Faint growth. Indol reaction negative	No growth	Precipitation of casein and separation of whey.

„	VII.	Soil	Flocculent growth throughout gelatin; no pellicle. Liquefaction, 4th day	Dry frondlike growth	No perceptible growth	Vigorous, moist brick-dust-coloured growth	Solid curd, no whey.
„	VIII.	Soil	Fine flocculi throughout gelatin; copious deposit	Pale lemon-coloured growth of cream-like consistence	Faint general turbidity. Indol reaction positive	Distinct moist cream-coloured growth	Precipitation of casein and separation of whey.
„	IX.	Soil	Gelatin remains clear; smooth surface pellicle	Diffuse growth, granular appearance; crusty and white in old cultures	Flocculent deposit; broth clear, no pellicle. Indol reaction positive	Snow-white dry crusty growth	Precipitation of casein and separation of whey.
„	X.	Soil	General turbidity and deposit. Liquefaction, 3rd day	Dry enamel-like palmate growth	General turbidity. Indol reaction negative	Dry, felt-like reddish-brown growth	Solid curd, no whey.
„	XI.	Soil	Stringy growth, mainly as deposit, which breaks up on shaking. Liquefaction, 7th day	Diffuse yellowish-white growth	General turbidity; slight deposit. Indol reaction positive	Moist brownish-yellow growth	Precipitation of casein and separation of whey.
„	XII.	Soil	General turbidity; finely divided deposit. Liquefaction, 4th day	Diffuse dry growth	General turbidity and deposit; fine pellicle. Indol reaction positive	Dry pipe-clay-coloured growth	Solid curd, no whey.
„	XIII.	Soil	Slight deposit. Liquefaction, 10th day	Dull yellow discrete colonies, with wavy margin	Flocculent growth. Indol reaction positive (without nitrite)	Moist yellowish-brown growth	Precipitation of casein and separation of whey.
„	XIV.	Soil	Diffuse flocculent growth	Rapid diffuse growth	General turbidity and deposit. Indol reaction positive	Moist slate-coloured growth	Precipitation of casein and separation of whey.

TABLE II.

Illustrating Range of Temperature for Growth of Thermophilic Bacteria.

Temperature = Soil	37° C.			40° C.			42·5° C.			45° C.			46-47° C.			51° C.			55° C.			60° C.		
Organism.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.
Bacillus I.	M.G.	G.	O	M.G.	G.	O	M.G.	G.	O	V.G.	O	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, II.	O	O	O	O	O	O	G.	M.G.	O	G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, III.	O	O	O	O	O	O	G.	G.	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, IV.	G.	M.G.	O	V.G.	V.G.	O	M.G.	G.	O	V.G.	M.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, V.	G.	M.G.	O	M.G.	G.	O	M.G.	M.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, VI.	O	O	O	O	O	O	G.	G.	O	G.	G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, VII.	O	O	O	G.	O	O	M.G.	M.G.	O	M.G.	G.	?	M.G.	V.G.	V.G.	V.G.	V.G.	?	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, VIII.	G.	O	M.G.	M.G.	O	M.G.	M.G.	M.G.	M.G.	V.G.	M.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	G.	G.	V.G.
,, IX.	O	O	O	M.G.	O	O	M.G.	M.G.	O	V.G.	V.G.	O	V.G.	G.	G.	V.G.	?	G.	V.G.	V.G.	G.	V.G.	V.G.	G.
,, X.	O	O	O	O	O	O	G.	O	O	G.	O	O	G.	G.	G.	G.	?	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XI.	O	G.	O	O	G.	O	O	G.	O	O	G.	O	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XII.	O	O	O	O	O	O	G.	O	O	G.	O	O	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XIII.	O	O	O	O	O	O	G.	O	O	V.G.	O	O	V.G.	V.G.	O	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XIV.	O	O	O	M.G.	M.G.	G.	V.G.	V.G.	O	V.G.	V.G.	G.	V.G.	V.G.	?	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.

Temperature = Soil	65° C.			67° C.			68° C.			69° C.			70° C.			72·5° C.			74·5° C.		
Organism.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.
Bacillus I.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
,, II.	V.G.	V.G.	O	V.G.	V.G.	O	G.	G.	O	O	O	G	O	O	O	O	O	O	O	O	O
,, III.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	O	G.	G.	O	O
,, IV.	V.G.	V.G.	O	V.G.	V.G.	O	O	G.	O	O	O	O	O	O	O	O	O	O	O	O	O
,, V.	V.G.	V.G.	O	V.G.	V.G.	O	?	V.G.	O	G.	G.	O	G.	G.	O	O	O	O	O	O	O
,, VI.	V.G.	V.G.	O	?	?	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
,, VII.	V.G.	V.G.	G.	V.G.	V.G.	G.	V.G.	V.G.	G.	V.G.	V.G.	G.	V.G.	V.G.	G.	G.	V.G.	G.	G.	G.	G.
,, VIII.	O	O	V.G.	G.	G.	G.	O	O	G.	O	O	G.	O	O	O	O	O	O	O	O	O
,, IX.	V.G.	V.G.	V.G.	O	V.G.	V.G.	O	O	V.G.	O	O	O	O	O	O	O	O	O	O	O	O
,, X.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	G.	G.	V.G.	G.	G.	O	O
,, XI.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	O	G.	G.	O	G.	?
,, XII.	V.G.	V.G.	G.	V.G.	V.G.	V.G.	G.	G.	V.G.	G.	O	G.	O	O	O	O	O	O	O	O	O
,, XIII.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	G.	V.G.	G.	G.	O	O
,, XIV.	V.G.	V.G.	?	V.G.	V.G.	O	O	V.G.	O	O	G.	O	O	G.	O	O	O	O	O	O	O

N.B.—G. = Faint growth. M.G. = Medium growth. V.G. = Vigorous growth.

A. = Nutrient agar. P.A. = Salt potato agar. P. = Potato.

indicates the relative vigour of growth at the various temperatures.

It will be seen that all the organisms described grew best at thermophilic temperatures. On some of the soils a slow and moderate growth took place, in a few instances at 37° to 40° C., and still more gave evidence of growth at 42° to 47° C. We may, however, judging by our observations take 55° to 65° C. and even to 67° C. as an optimum temperature, both as regards the quickness and the amount of growth that occurred. It was interesting to note that in one or two instances a very good growth occurred at 72·5° C., whilst in a few cases evidence of growth was obtained even at 74·5° C. Another interesting point was the remarkable range of temperature at which growth could occur. Thus the Bacillus VIII. showed evidence of growth from 37° C. up to 69° C., whilst Bacillus XI. developed from 37° C. up to 74·5° C.—a remarkable range. At the same time there was no doubt about the optimum temperature being above 50° C.

The suggestion has been made that the phenomenon is simply an instance of ordinary saphrophytes adapting themselves to an abnormal temperature. It is difficult to believe that this is the case. Such adaptiveness or "anpassung" requires time and a careful training of the organism under ordinary circumstances. The growths were too good and rapid to lend support to such a theory. However they may live under ordinary circumstances, there is no doubt with regard to the favouring influence of the factor, temperature. At the same time the varied conditions that must present themselves in nature may favour their growth, even if it is slow, *e.g.*, certain chemical conditions, not represented by the culture soils of the laboratory, of which we are at present ignorant.

The suggestion that it is a question of adaptivity on the part of ordinary bacteria led to a series of experiments with some well-known forms—we had already proved that the ordinary saprophytes will not grow at the optimum thermophilic temperature.

The following short table (III.) gives the results obtained.

The conclusion arrived at was, that as regards conditions of temperature the mesentericus group of organisms appears

TABLE III.

Temperature range of typical saprophytic organisms.

Organism.	Temp. 37° C.	Temp. 40° C.	Temp. 42·5° C.	Temp. 45° C.	Temp. 46-47° C.	Temp. 50° C.
B. mesentericus vulgatus ..	+	+	+	+	+	+
,, ,, niger	+	+	+	+	+	+
,, vermicularis	+	+	+	+	+	+
,, mycoides	+	+ ?	O	O	O	O
,, ramosus	+	+ ?	O	O	O	O
Proteus vulgaris	+	+	+ ?	+ ?	O	O
S. pastorianus II.	+	+ ?	O	O	O	O
,, ellipsoides I.	+	+ ?	O	O	O	O

to form a connecting link between the ordinary saprophytic and the thermophilic bacteria.

As regards optimum temperature, the micro-organisms commonly fall into three great groups—

(1) For the great majority of saprophytic bacteria an optimum of 15° to 20° C..

(2) For the parasitic forms an optimum of 37° C.

(3) For the thermophilic organisms an optimum of 55° C. or thereabouts.

The facultative parasites, such as the bacillus anthracis, form a link between the first two groups, and grow well at the respective temperatures. The mesentericus group connect the second and third group.

Anaerobic Experiments.

The organisms isolated had hitherto been studied under aerobic conditions, which appeared in every way the most suitable for their growth. A series of experiments was made to discover whether the pure strains isolated could grow anaerobically, and also whether by direct inoculations with soil the existence of anaerobic forms could be proved.

The anaerobic cultures (hydrogen atmosphere) made directly with soil in broth gave an abundant growth of slender bacilli. The broth had a foul odour, and became blackened. Deep cultures of soil in potato agar gave a slow development of colonies. In one instance subcultures were made from the broth tubes in the depth of agar tubes, and the surface sealed with gelatine. Colonies appeared below the surface, and a certain amount of gas and a butyric odour were developed. All anaerobic inoculations of soil in broth at thermophilic temperatures gave a growth and offensive odour. There were therefore forms capable of growth under anaerobic conditions.

A number of pure cultures of the organisms were placed in gas jars containing an alkaline solution of pyrogallic acid, and a certain number in sealed Hueppe's tubes. These experiments did not succeed, and led to the conclusion that the thermophilic bacteria we had isolated require oxygen for their growth. All the tubes on being placed in free contact with air developed growths. Experiments were likewise made with deep cultures in the salt potato agar. No growth occurred along the line of the stab, but in each case a good surface growth occurred in contact with the air. Like the growths on potato, the surface growths on potato agar developed various pigments—yellow, brown, pink, &c.

The growths that do occur (*e.g.*, from soil), under anaerobic conditions require further investigation, and it is hoped to deal more exhaustively with this subject in a later paper on the cellulose fermentation.

Rabinowitsch states that the forms she isolated were facultative anaerobes, and that at low temperatures they frequently grow quicker anaerobically, especially in broth. This suggests the possibility of an anaerobic growth of these organisms in nature at ordinary temperatures.

To test these suggestions we made deep cultures in sugar agar, and kept them at blood-heat for about three months, but there was practically no deep growth and only a trace of surface growth in two instances. The tubes, however, on being placed at 55° C. immediately developed growths which were mainly on the surface.

"Shake" cultures in ordinary agar at 22° and 37° C. likewise gave negative results.

Broth cultures in hydrogen showed no growth after ten weeks at blood-heat. The tubes were then opened so as to admit air, and placed at 55° C., when a good and immediate growth occurred in every instance. Further experiments always gave the same results, whether carried out with fluid or solid media. The results were, therefore, negative as regards an anaerobic growth of the organisms at the lower temperatures. Rabinowitsch's results are not decisive, and do not appear to have been of a uniform character, as it is stated that at times no growth occurred for some unexplained reason. We do not think that this theory will explain their active existence in nature under the ordinary conditions of temperature. It is, however, but reasonable to suppose that such a widely distributed group of organisms must find favouring natural conditions outside the factor of temperature, these conditions being probably of a chemical nature. We are equally ignorant of the life conditions of many pathogenic organisms outside the body. At the same time, distinct evidence was obtained of the anaerobic existence of certain thermophilic forms at high temperatures.

Action of Light.

A series of cultures were exposed on the roof during sunny weather during a period of twelve days. There was some evidence of growth in two cases. On placing the tubes at 55° C. they remained sterile with two exceptions. Of the fourteen organisms twelve had been killed by the direct sunlight.

Variations in Form.

A number of experiments were made to test the effect of temperature in producing any possible variations in form of the organisms. Three typical bacilli were cultivated at gradually increasing temperatures, viz., from 40° C. up to 70° C. The results may be shortly summarised as follows: —At the lower temperatures bacilli in short or long chains

were generally obtained. At the thermophilic temperatures the tendency to thread formation became more marked, as well as a tendency to develop interlacing cladothrix-like filaments without, however, a genuine branching. The organisms always retained the character of bacilli at the various temperatures.

Action on Culture Media.

(1) *Reaction.*—The organisms were capable of growth in sugar broth and litmus broth.

In the first experiments made the sugar broth became acid at the end of six days, whilst a bleaching generally occurred in the simple litmus broth.

A sugar-free peptone beef broth was made from stale meat and carefully neutralised. The fourteen organisms were cultivated in this broth at 55° C. No acid was detected in the sugar-free broth and the tubes gave a more or less alkaline reaction. The tendency appeared to be in the direction of alkali formation.

In litmus agar the organisms produced a bleaching of the litmus in twenty-four to forty-eight hours with one doubtful exception.

In sugar agar a growth appeared in each instance, though more slowly than in a non-sugar soil. No gas development took place.

On sugar litmus agar after forty-eight hours Bacillus X. and Bacillus XII. showed a reddening of the litmus, the others showed slight alkalinity or no distinct change occurred.

(2) *Reducing properties.*—The nitrate solution used had the following composition:—Potass. phosph., 0·1 grm.; magnes. sulph., 0·02 grm.; calc. chlor., 0·01 grm.; potass. nit., 0·168 grm.; dextrose, 0·3 grm.; peptone, 0·25 grm.; aq. d. 1000 grm.; and calc. carb., 4 grms. This fluid was inoculated with pure cultures and incubated at 55° C. and finally tested with Nessler's reagent. The control tubes tested for nitrites and ammonia gave negative results, whilst tubes inoculated directly with soil gave a good reaction. As regards the pure cultures of the organisms, the results with

Bacilli I., IV. and V. were negative. Bacilli II., III., IV. and XI. gave very good reactions and fair reactions were given by the remaining organisms. The positive tubes on testing with a solution of sulphanilic acid gave the nitrite reaction, showing that a reduction had taken place. The above experiments were repeated with similar results, on testing with Nessler's reagent and sulphanilic acid. The majority of the organisms, therefore, possessed reducing properties.

(3) *Action on proteids.*—It was already noted that a certain number of the organisms produced a liquefaction of gelatin.

A series of tubes of inspissated *serum* were inoculated directly with soil and incubated at 55 to 65° C. A complete liquefaction of the serum was produced and a most offensive smell. The action of pure cultures of the organisms on serum were tested under the same conditions. The growths were slower than on the ordinary culture media.

At the end of four days eight of the bacilli gave a medium or good growth on the surface of the serum without any evidence of liquefaction. The remaining six bacilli gave good growths accompanied by a pitting or complete liquefaction of the serum, viz., Bacilli I., II. and XIII. produced pitting and Bacilli VII., IX. and XI. distinct liquefaction of the serum.

Flasks containing *meat* with sterilised humus and a small amount of peptone water were inoculated aerobically with the mixed growths obtained by sowing soil on blood-serum. The meat underwent an active decomposition through the action of the mixed growths.

Another series of flasks was inoculated with pure cultures of the bacteria, but, although a slight odour was evolved, no results were obtained to compare with those due to the mixed growths as contained in soil.

Blood albumen, inoculated directly with soil, gave vigorous growths, along with an active decomposition and a most repellent odour. The pure cultures, again, did not give results corresponding in intensity or completeness. In some instances no effect was produced; in other instances a slight

disintegration of the albumen took place, with a putrid, a mousey, or an aldehyde odour.

Eggs, inoculated with soil, developed a distinct smell, and their contents were blackened. With pure cultures a growth occurred in the eggs, but the natural mixture of organisms always gave better results.

It would, of course, be advisable, in this connection, to make anaerobic experiments as well, but there was no doubt as to the decomposition of proteid material by these organisms, especially in mixed cultures obtained directly from soil. The action appeared to be of a symbiotic nature.

(4) *Action on Carbohydrates.*—The organisms, in pure culture, were added to a 2 per cent. solution of *cane sugar* in peptone water at first, but finally the sugar solution was used alone, and was found to be preferable. The tubes were incubated at 55° C. Eight of the organisms were found to invert cane sugar, viz., Bacilli III., VI., VII., IX., X., XI., XIII., and XIV. The organisms were further grown on a *starchy* soil at 55° C. The starch was diastased by four organisms, viz., Bacilli IV., IX., X., and XIII. The organisms multiplied in the starch solution, which became yellow on adding potash and reduced Fehling's solution.

The thermophilic bacteria had, therefore, an inverting and diastatic action at these high temperatures. Dr. Oprescu found that two of the five bacilli he examined exhibited both a proteolytic and a diastatic action. The action of the proteolytic enzyme ceased at 60° to 65° C., but the diastatic was more resistent, as one hour at 65° C., and even one hour at 80° C., did not inhibit its action.

Experiments with Ensilage.

Generally speaking, ensilage is a process for the preservation of green fodder, in which the fodder is so packed that, by dryness and the absence of air, the acetic acid fermentation is hindered, and the lactic acid fermentation is favoured. As is well-known, the "silos" attain a considerable temperature. It was therefore of interest, in this connection, to examine samples of the silos. The first samples were

obtained from Devonshire; the temperature inside the stack was not known, but the edge was 78° F. at the time of removal. Samples of the straw at 60° C. gave growths in every instance, and plate cultures were made from these mixed growths. It was noticed that in the original tubes a second series of growths had started, of a whitish colour, on the straw itself. These growths appeared more slowly, and appeared to prefer the straw to the agar as a soil. We noticed growths of similar appearance in the course of the decomposition of cellulose to be referred to subsequently.

A second sample from a stack (temperature 50° C.) yielded quick and slow growths, which appeared after six days. Microscopically, a large number of bacilli were observed.

A sample of ensilage was placed in a tin box, and hermetically sealed, and incubated at 60° to 65° C. When opened at the end of five weeks it was found to be still moist and steaming, and to be covered, in parts, with a whitish growth. The whole of the ensilage was swarming with living bacilli; and numerous spores were present. Cultures were made at 22° C. and 37° C., with negative results. With cultures kept at 60° C., a copious growth was obtained within eighteen hours. The ordinary saprophytes had been killed, and only the thermophilic bacteria had remained alive and active. The fodder kept in the box at 60° C. for two months gave copious growths, at the end of that time, of thermophilic bacteria, and no growths, either aerobic or anaerobic, occurred at 22° C. and 37° C. A hanging drop showed many bacilli, including motile forms. It was most interesting to find that after two months at 60° C., the ensilage was swarming with active bacteria. There could not be a more conclusive experiment as to the favouring conditions of a high temperature on these forms of life.

Experiments with Cellulose.

The occurrence of thermophilic bacteria in ensilage, and their relation to the spontaneous heating of hayricks led us to consider that these organisms probably exerted some action on cellulose. The first experiment made was quite

unexpectedly successful. To a solution of nutrient salts containing a trace of broth, were added Swedish filter paper and a small quantity of soil. The mixture was incubated at at 60° C. The result was an active development of gas and smell. In about ten to fourteen days the filter paper was completely broken up. A microscopical examination on the fourteenth day showed numerous bacilli, some of which were sporing. There were also small bacteria present embedded in a zoogloea—an undoubted disintegration of the cellulose had occurred. We repeated these experiments with filter paper, and also with samples of pure cellulose, for which we are indebted to Mr. Cross. A series of tubes, after inoculation with soil, were incubated at 22° to 37° and 60° C. In one series the tubes were aerobic, in another anaerobic. The fermentation of the cellulose started slowly and first became marked about the fourteenth day. A complete decomposition of the cellulose occurred at the end of three weeks at 60° C., whilst at 22° and 37° C. no action was evident. The results were so striking that it was felt advisable to have a control analysis made, and this was very kindly carried out by Mr. Cross. The cellulose used consisted of prepared films of cellulose hydrate, as obtained from the solution of the original fibrous cellulose (cotton) in the form of thiocarbonate. The samples represented a chemically pure cellulose of the normal group in its most reactive condition (Cross). We also obtained from Mr. Cross specimens of pure straw and esparto cellulose.

Mr. Cross reported as follows on the solutions, which showed a disintegration of cellulose by the thermophilic organisms:—

"(1) No reduction CuO, in original, nor after boiling with acid.

"(2) No other evidence of any proximate products of resolution, *i.e.*, carbohydrates of dimensions, $N.C^6$.

"(3) On distillation, 25 c.c. gave volatile acid = 1 c.c. of normal NaOH, containing acetic and butyric acids. Residue gave traces only of furfurol on distillation with HCl (1·06 s.g.).

"It appears that the destruction has been for the most

part complete, probably to CO_2 and CH_4. On further investigation you may be able to get an intermediate stage or an organism acting less severely."

These experiments therefore gave undoubted evidence of the digestion of cellulose by thermophilic bacteria.

The experiments were continued in the following manner:—A series of flasks was prepared containing pure cellulose and Naegeli's nutrient solution + 5 c.c. of nutrient broth, and a second series containing broth alone and cellulose. These were inoculated with soil and kept at 60° C. After about three days an active fermentation started in the flasks of cellulose and broth, but had not yet appeared in the flasks without broth. The traces of broth appeared to help the fermentative process.

Anaerobic experiments were also made in Hueppe's tubes containing Naegeli's solution, broth, pure cellulose and a small quantity of soil. After nine to ten days the cellulose was attacked in the anaerobic, as well as in a series of aerobic tubes, but appeared to be more advanced in the anaerobic tubes. A marked smell occurred in both instances. A large number of bacilli were present, and we specially noted that several new forms appeared that had not been noted in the experiments without cellulose.

A further series of experiments with well washed esparto cellulose resulted in the disappearance of the cellulose at the end of three weeks at 60° C. In this instance a mixed culture from fermented cellulose had been added. The control tubes kept at 22° to 37° C. were not visibly affected after four weeks.

The rate of fermentation and disintegration of the cellulose varied from seven to twenty-one days as a general rule.

All the above results were brought about by mixtures of thermophilic bacteria, occurring naturally in the soil and the action appeared to be of a symbiotic nature. Their action resulted in a *complete disintegration* of filter paper, fibrous cellulose and esparto cellulose.

The further discussion of the fermentation of cellulose must be left for a subsequent paper.

So far, these experiments, whether with proteids or cellulose, always gave the best results when a mixture of organisms was present. It may also be that the addition of soil supplied valuable constituents for their growth, and that decoctions from soil may prove of help in the future study of these organisms.

General considerations.

Without considering any possible chemical or other favouring conditions that may exist, in how far can such organisms find in nature the temperatures that are so well suited for their growth? It was suggested in our first paper that in the course of many natural fermentations the heat developed frequently reached a temperature suitable for thermophilic bacteria; we have since then looked into the subject, and obtained some direct evidence on the point.

The following are readings taken in a *silo* :—August 22, 120° F.; 23rd, 150° F.; 24th, 140° F.; 25th, 130° F.; 26th, 160° F.; 27th, 160° F; 28th, 150° F. The silo remained at 150° F. for two months.

A pit of stable manure gave following readings:—At 1 ft., 60° C.; 2 ft., 65° C.; 3 ft., 62° C.; 6 ft., 70° C.; and a heap of fresh manure after twelve hours rose to 50° C.

A heap of field manure at the surface was 40° C.; 1 ft. deep, 60° C.; 3 to 4 ft. deep, 70° C.

Refuse moss litter gave temperature of 42° to 60° C.; and straw litter 51°, 75°, 83° and 84° C. A small heap of hay saturated with urine and fæces had a maximum temperature of 71° C.

The temperature of a heap of straw litter during six days is given in the following table :—

	1st day.	2nd day.	3rd day.	4th day.	5th day.	6th day.
2 feet	63° C.	60° C.	60° C.	60° C.	63° C.	50° C.
3 „	78° C.	75° C.	70° C.	71° C.	75° C.	72° C.
5½ „	82° C.	85° C.	83° C.	81° C.	84° C.	87° C.

These temperatures are, of course, mostly favourable for the development of thermophilic organisms.

The following temperatures have been noted in other fermentations, associated with bacteria:—

Spontaneous heating of hay and cotton	67° C.
Germinating barley	60° C.
Tobacco fermentation	54° C.
Snuff „	50° C.
Air-dried hay	70° C.
Brown hay	70° C.
Lactic acid fermentation	52° C.
Butyric acid fermentation	40° C.

The spontaneous heating of hops is associated with an organism, the bacillus lupuliperdi. There is a wide and profitable field of research open with regard to the fermentations that occur at 50° to 60° C. In the spontaneous heating of hay ricks the temperature rises to 50° to 60° C. through the agency of bacteria, and chemical processes are thereby started which lead to the production of further heat and inflammable bodies.

The enormous amount of manure that is constantly undergoing decomposition must furnish a most favourable nidus for thermophilic organisms, and we are sometimes apt to forget the amount of heat that is continually being developed at all seasons of the year by the processes of nature.

The forms of life we have been studying constitute a large and important class of organisms endowed with a marked vital activity, which finds its most active expression at thermophilic temperatures, as is evidenced by the fermentative and other changes they are capable of bringing about under such conditions. We have a most striking example of this in the complete disintegration of cellulose under their influence. The exact conditions that may probably favour their growth at subthermophilic temperatures are not yet known—they may possibly be of a chemical nature.

REFERENCES.

(1) Macfadyen and Blaxall. "Thermophilic bacteria," *Journal of Path. and Bact.* November, 1894.

FIG. 1

FIG. 2

FIG. 3

FIG. 4

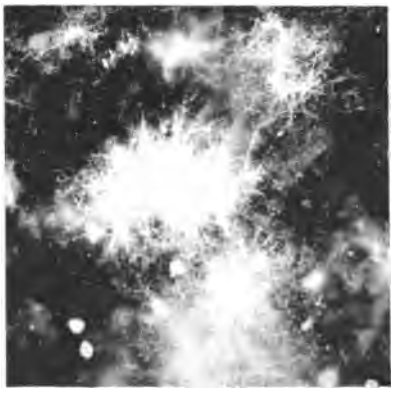

FIG. 5

FIG 6

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FIG. 7

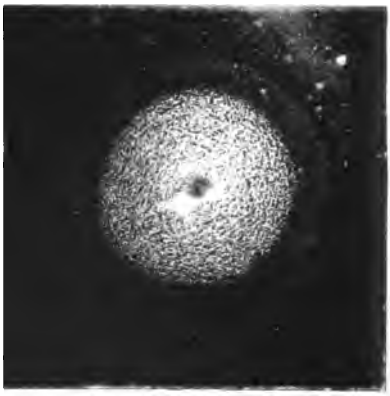

FIG. 8

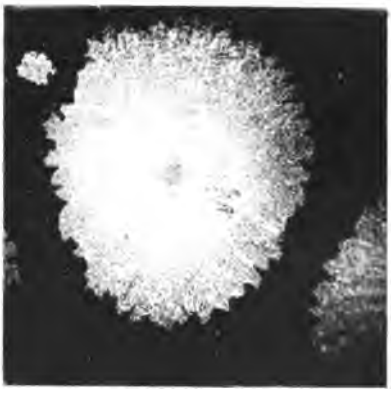

FIG. 9

FIG. 10

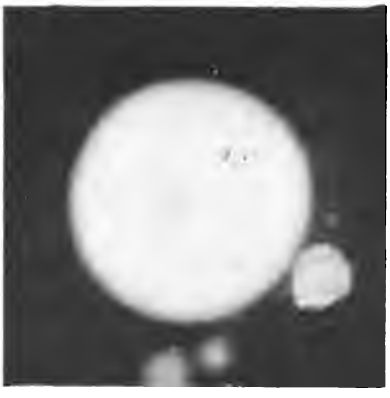

FIG. 11

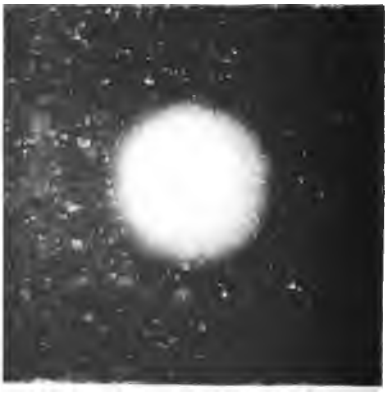

FIG. 12

Photoprint, Bale & Danielsson, Ltd.

THERMOPHILIC BACTERIA

FIG. 13

FIG. 14

FIG. 15

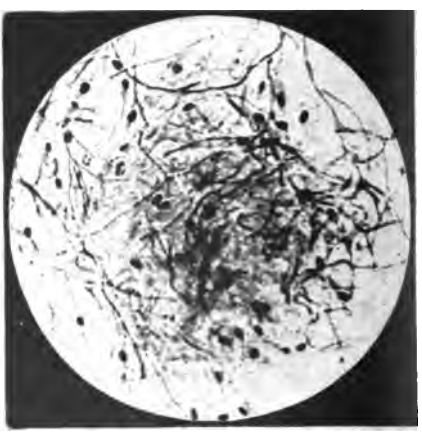

FIG. 16

Photoprint, Bale & Danielsson, Ltd.

(2) Rabinowitsch. "Thermophile Bakterien," *Zeitsch. f. Hyg.* Bd. xx., 1895.

(3) Teich. "Beitrag z. Kenntniss thermoph. Bakterien," *Hyg. Rundschau.* 1896.

(4) Laxa. "Thermoph. Bacillus aus Zuckerfabrik produkten," *Centralb. f. Bakt.* Abth. ii., bd. iv., 1898.

(5) Schillinger. "Thermoph. Bakterien," *Hyg. Rundschau.* 1898.

(6) Kidzior. "Thermoph. cladothrix," *Arch. f. Hyg.* Bd. 27, 1896.

(7) Oprescu. "Thermoph. Bakterien," *Arch. f. Hyg.* Bd. 33, 1898.

(8) Häpke. "Selbstentzündung d. Heu," *Abhand. d. Naturwiss.* Verein, Bremen.

(9) Tsiklinski. "Les mucèdinèes thermophiles," *Annales de l'Instit. Pasteur.* Vol. 13. November, 1899.

DESCRIPTION OF PLATES.

Fig.	1.	Bacillus	I.	(from dejecta) .. Plate colony.
,,	2.	,,	II.	(,, ,,) .. ,, ,,
,,	3.	,,	IV.	(,, ensilage) .. ,, ,,
,,	4.	,,	V.	(,, Thames mud) ,, ,,
,,	5.	,,	VI.	(,, Plymouth seawater) ,, ,,
,,	6.	,,	VII.	(,, soil) .. ,, ,,
,,	7.	,,	VIII.	(,, ,,) .. ,, ,,
,,	8.	,,	IX.	(,, ,,) .. ,, ,,
,,	9.	,,	X.	(,, ,,) .. ,, ,,
,,	10.	,,	XII.	(,, ,,) .. ,, ,,
,,	11.	,,	XIII.	(,, ,,) .. ,, ,,
,,	12.	,,	XIV.	(,, ,,) .. ,, ,,
,,	13.	Thermophilic bacillus.		Growth at 55° C.
,,	14.	,,	,,	,, ,, 65° C.
,,	15.	,,	,,	Sporing Stage.
,,	16.	,,	,,	Cladothrix-like growth, at maximum temperature; numerous spores.

The plate colonies and cultural peculiarities present the best means of differentiating the above forms.

THE TECHNICAL APPLICATIONS OF BACTERIOLOGY.

BY G. HARRIS MORRIS, PH.D., F.I.C.

It was the close association of the fermentation industries with the district of which Lille was the centre that turned Pasteur's attention to those studies which established the true nature of the phenomena in which micro-organisms play a part. It is unnecessary to recapitulate the views on fermentation which were held from forty to fifty years ago; it is enough to say that the relation of the micro-organisms present in any given fermentation to the results obtained was not recognised. Pasteur first investigated the lactic fermentation, and showed the connection between the so-called *levure lactique* and the production of lactic acid from carbohydrates; then, a few years later, he had his attention turned to the manufacture of vinegar, of which Orleans was then the centre, and demonstrated the causal relationship of the acetic ferment to the manufacture of vinegar, and indicated the conditions which have a favourable or unfavourable influence on the acetifying action of the bacteria. These researches were followed up by investigations of even greater practical importance, namely, those on the diseases of wine and on the silkworm disease. Both these industries were of immense national importance to France, and the yearly loss which followed defects in the wine, on the one hand, and the disease or death of the silkworms, on the other hand, could hardly be computed. Dealing in the first place with the diseases of wine, Pasteur showed that these were due to the presence of bacteria in the defective samples, and that they could to a large extent be prevented by adopting a system of partial sterilisation, which we now know as *pasteurisation*, and which is now applied on a commercial scale to other

liquids besides wine. The silkworm disease, which Pasteur was then called upon to investigate, although of an entirely different nature to those affecting wine and vinegar manufacture, was then approached from an industrial standpoint, as it entailed a yearly loss of upwards of four millions sterling. Pasteur was able to demonstrate the cause of the disease and to propose remedies to meet it, and this investigation undoubtedly formed the stepping-stone to the later researches in the domain of pathology, with which we are not now concerned.

But the industrial process with which Pasteur's name is most widely known is the manufacture of beer. Led to examine this process by the results of the war of 1870, Pasteur showed the great influence which the presence of bacteria exerts on the character of beer containing them. Various diseases which beer from time to time exhibits, such as ropiness, acidity, &c., were proved to be due either to the use of yeast containing certain bacteria, or to contamination of the wort by the bacteria in question at some stage of the process. Pasteur's work on this subject is contained in his well-known book, "Etudes sur la Bière," the publication of which led to a revolution in the practice of brewing. In this book Pasteur describes the organisms producing the different defects of beer, and describes the method he adopted for obtaining yeast free from bacteria. He also gives a method by which yeast can be kept pure on a practical scale by treatment with a solution of tartaric acid, and he states that if the sterile wort coming from the copper is only allowed to come in contact with pure air during cooling and fermentation, is fermented with pure yeast, and is stored in casks or other vessels freed from disease germs, no changes of an injurious nature will take place in the beer.

The researches of Pasteur, then, established the fact that in certain industrial processes of fermentation, certain distinct organisms were concerned, and that in order to get the best results, conditions must be adopted which will keep the fermenting liquids free from other organisms, so far at least as can be ascertained from microscopical examination. Furthermore, in processes of alcoholic fermentation, the "pure

yeast" he advocates is simply yeast free from bacteria or other organisms which can be detected or distinguished by the microscope.

A new departure was, however, made by Prof. E. C. Hansen, when in 1882 he published his experiments on the culture of yeast from single cells. Practical experience in connection with brewing showed him that, although a yeast might be microscopically pure, yet it by no means followed that it would give good results in the brewing. This led to the conclusion that the difference must lie in the yeast cells themselves, and apparently similar cells might belong to different species, giving different results in practice. From this, as a starting point, Hansen demonstrated that some of the most serious and commonest diseases of beer were caused not by bacteria, but by certain species of yeast, and, therefore, that it was not sufficient to purify yeast from bacteria and other foreign organisms. Further experiments on a practical scale established the fact that different species of good brewing yeast could produce beers of essentially different character, so that it is necessary not only to eliminate the bacteria and disease yeasts, but to select the type of good or natural yeast most suitable for any particular brewing.

In order to make the selection, reliable methods for the preparation of pure cultures were necessary, and these Hansen elaborated. Lord Lister had used a dilution method in 1878 for the purpose of obtaining a pure culture of the lactic ferment, and Hansen first adopted this with certain modifications which made the method more exact. The number of yeast-cells contained in a certain volume of liquid was determined by means of a hæmatometer and a cover-glass divided into squares, the dilution generally aimed at being one cell in each cubic centimetre of the mixture. The dilute yeast was then introduced into flasks containing sterilized beerwort, in such a quantity that only a few of the flasks were infected, and, after thorough shaking to distribute the cell or cells in the wort, the flasks were allowed to remain quite quiescent until some growth became visible. It was assumed that the yeast-cell or cells would, after agitation, settle to the bottom of the vessel, and give rise to a separate

yeast speck; if a flask showed only one such yeast speck, it was considered that it contained a pure culture from a single cell, if it contained more than one speck it was rejected. This, in brief, was the method by which Hansen carried out his earlier work, and which he claims to have first fulfilled the requirements of an exact dilution method. It depends entirely on the use of liquids, plate-culture by the aid of nutrient gelatine not having then been introduced. Solid substrata had been used by Brefeld and others for morphological investigations, but it was Koch who first introduced the methods of plate-culture now in use. In his earlier work he obtained his pure cultures by means of streak cultures in nutritive gelatine, but later he improved this, and after mixing a dilution of the organism with fluid, sterile, nutritive gelatine, and agitating the mixture to distribute the cells, he poured the still fluid gelatine on to a sterile horizontal glass plate, and allowed it to set. Hansen, however, considered that this method was not sufficiently accurate, and says, " in order to obtain an absolutely pure culture with certainty, it is necessary, even when the gelatine method is employed, to start from a single cell." With this end in view he combined Koch's method with the use of the moist chamber, and after making a dilution of the yeast, first with sterile water, and then with nutrient gelatine (in this case beer-wort gelatine), in the usual way, a little of the wort gelatine containing the yeast-cells is spread on the under side of the cover-glass of the moist chamber. When the gelatine has set, the moist chamber is placed under the microscope, and the position of those cells which can develop into colonies without coming into contact with other colonies is accurately marked. Only those colonies which can be proved by direct microscopical examination to have developed from single cells are then inoculated into a liquid nutrient medium.

The justness of Hansen's contention that the method of Koch does not always ensure absolute purity, is shown by the fact that one observer, working with bacteria, obtained 134 different bacteria from 100 colonies prepared by the method, whilst another, working with yeast and wort

gelatine, found that, on an average, 108 yeast cells only gave rise to 100 colonies.

Such was the method adopted by Hansen to obtain pure cultures of yeast, in order to prove his conclusion that many of the defects of beer are due to the presence of yeast cells, which cannot be microscopically detected when mixed with healthy and normal yeast. Another and most important point was also established, namely, that, putting aside the question of diseases of beer, in order to obtain the best results it is essential to make a careful selection of the species or race of yeast with which the fermentation is to be carried on. It is this principle which has had such a wide-spread influence on industrial processes in which micro-organisms are concerned.

As might be expected Hansen's views were at first objected to by brewers to whom he mentioned them, but he was able to show their correctness on a practical scale in two breweries in Copenhagen; in one, the beers had suffered for some time from yeast turbidity, with very disastrous results; and in the other, the beer acquired an unpleasant odour and bitter taste. Both these cases were investigated by Hansen, and he was able to conclusively show that the maladies were not produced by bacteria, as was at first supposed, but were due to the presence in the brewery yeast of wild yeasts, which gave rise to the above-mentioned effects. The second of the breweries was that of Old Carlsberg, then owned by the late Captain I. C. Jacobsen, and which has become so well known in connection with Hansen's famous discoveries. The importance of the new departure having been thoroughly established, Jacobsen extended the system to the whole of his brewery, and from that time (1884) to this the fermentations in the Old Carlsberg brewery have been conducted with specially selected pure yeast cultures, with, it is understood, the most excellent results. In spite of much opposition, to which it is unnecessary to refer, the system gradually spread to other breweries in Denmark and on the Continent, and at the present time it is more or less extensively used in nearly all the European beer-producing countries, in North and South America, in Asia, and in Australia.

The practical advantages resulting from the use of a selected pure yeast are, to use Hansen's words:—

(1) A definite result and rational working are secured where formerly everything depended more or less upon chance.

(2) Maladies in the beer, causing great losses, are guarded against.

(3) A yeast is obtained having a higher commercial value than the ordinary impure yeast.

(4) Finally, it helps to raise the industry, and this must be a point of great interest to the intelligent brewer.

In practice, there are naturally several points to be considered, such as the selection of the yeast type most suitable for the particular brewery, the steps to be taken to keep the culture pure, the means to be adopted to keep a constant supply of the same yeast type, &c.; but these are matters which need not be dealt with here. It is sufficient to say that these practical difficulties can be, and have been, surmounted in those breweries in which the system is at work.

The application of pure cultures to brewing has been thus fully dealt with, as it was in this connection that the use of selected types of micro-organisms had its origin; and before proceeding to mention some of the other technical applications of the system, it will be well to say a few words on the position of pure yeast culture in English breweries. When Hansen first attempted to put his ideas into practice, the objection was raised in Denmark and elsewhere that the use of a culture derived from a single cell would interfere with what is known as the after, or secondary, fermentation of beer; but it would appear that this objection was ill-founded in connection with the systems of brewing adopted on the Continent. The same doubts were expressed with regard to its application to English brewing, and, although it is claimed that, as on the Continent, the question has been satisfactorily answered, yet, in the writer's opinion and experience, this is not the case; and we still await the solution of the question of the successful application of pure yeast culture to the conditions of English brewing. It has been suggested that the solution lies in the direction of the

employment of two or more selected races of yeast—one to carry on the primary fermentation, and the second the secondary fermentation; and many breweries have been working, with more or less success, on these lines. The fact, however, remains that, up to the present, no satisfactory system, capable of general application, has been devised; and the question still awaits solution. That the use of pure yeast, in some form or other, has a future before it, no one can doubt; but the conditions of brewing, which will allow of that employment being successful, yet remain to be worked out.

Once the importance of the use of selected pure cultures had been demonstrated, the application to other industries speedily followed. The technical processes involving the use of yeast were, perhaps, the first to adopt the principle, and now in distilleries and yeast factories, in the production of wine and in cider-making, the system is well established. In distilling, especially, the use of selected races has been of advantage in decreasing the amount of acid formed during fermentation, and increasing the yield of alcohol and in decreasing the formation of by-products. Interesting results have also recently followed the combined use of pure cultures of a mould and a yeast. These remarks apply to the manufacture of ordinary alcohol or spirits of wine, but yeast selection has also been employed in connection with the manufacture of rum and arrack. In the preparation of pressed yeast or yeast for baking, the system of pure culture is also largely utilised on the Continent with excellent results. In English distilleries no attempt has yet been made, so far as is known, to employ pure yeast.

In the application of selected pure yeasts to wine fermentation, some remarkable and interesting results have been obtained. Until attention was directed to Hansen's methods in connection with brewing, the grape must was left to the spontaneous fermentation brought about by the yeast present on the surface of the grapes. The same thing occurs to a very large extent at present, but in some districts selected types of the wine yeast—*saccharomyces ellipsoideus*—are being used with great advantage. Investigations on

this point were carried out in France, Germany and Italy, and the results obtained show that the type of yeast used for the fermentation of the must has a very great influence on the bouquet and taste of the wine. In some quarters it was at first contended that the character of the wine was determined exclusively by the type of yeast, but this has been shown to be incorrect, and although the taste, bouquet and general character of the wine are influenced by the yeast, yet the composition of the must is of the first importance. The different races of yeast develop during fermentation different characteristic bouquets, in greater or less amount, and these are due to products which are produced during fermentation from the must. Institutions have been founded from which pure selected yeasts are supplied to the wine-growers, thus enabling them to produce better wines, both as regards taste and bouquet and freedom from the injurious effects produced by foreign micro-organisms. Another important point in the supply of selected yeasts is that a grower can obtain the same type year after year, and thus insure his produce having a constant character.

Advances in the same direction have been made in the cider and fruit-wine industries. The fermentations here have also hitherto been spontaneous, and investigations have shown that the yeast so produced is a mixture of bad and good types. Selection of those giving good results has led to a great improvement in the manufacture of cider and similar beverages, giving them increased soundness and a true vinous flavour. Considerable advances have also been made in the use of selected wine yeasts for cider fermentation. The results obtained are certainly very remarkable. The writer had a short time ago an opportunity of tasting cider manufactured in this way in Denmark; it had all the characteristics of a light hock. Cider-making is an important industry in some parts of England, and attempts have been made in Somersetshire on an experimental scale to conduct fermentations with pure yeasts. The writer has, however, been informed by Mr. Lloyd, who has had charge of the experiments, that many difficulties have been encoun-

tered in the matter, and that much has yet to be done before pure yeast can be employed on an industrial scale.

But the industries involving alcoholic fermentation are not the only ones which have derived benefit from Hansen's discoveries. In agriculture, in the manufacture of tobacco, in tanning, &c., the methods of pure culture have been adapted to the needs of the technologist.

The use of selected cultures has been applied in more than one direction in agriculture. Perhaps the most important is that of the dairy; here the souring of cream, the ripening of cheese, and the defects frequently exhibited by milk, butter, and cheese have all received attention. In Germany selected pure cultures of bacteria are being used for the first-named process, and steps are being taken to put the ripening of cheese on a sound basis. It has long been the custom to inoculate Roquefort and Edam cheeses with suitable micro-organisms—in the case of the former a mould, of the latter a bacterium—in order to ensure satisfactory ripening, and now the methods of pure culture are being applied to the manufacture of these and other classes of cheese with good results. The process of ripening is thereby more certain and of a constant character.

The fixation of nitrogen by bacteria and the discovery of the nodules in the roots of certain plants and their functions afforded another opportunity for the use of selected cultures, and such cultures are in fact being used in Germany on waste lands, which it is desired to bring under cultivation for nitrogen-collecting plants. The organisms concerned in ordinary nitrification have also been isolated and examined, and it is evident that there is a considerable field open here for selected cultures, especially in connection with the disposal of sewage.

In the manufacture of tobacco considerable progress has been made in the direction of the use of selected cultures. The tobacco leaf undergoes a process of fermentation, and it has been shown that the flavour of tobacco of different localities depends very much upon the bacterium flourishing in each particular locality. Pure cultures of several of these have been made and transplanted to other centres of tobacco

manufacture with excellent results. Thus German tobacco leaf has been fermented with the bacteria from the West Indies with the result that the former has been greatly improved as regards flavour. In America also the bacteria from the Cuban tobacco have been introduced with most satisfactory results, and as the soil in the tobacco-growing districts of Florida is similar to that in Cuba, it is anticipated that the introduction of the selected cultures will have a great influence on the tobacco manufacture of the former district.

The tanning industry has also received attention, and some important work has been done in England in this branch of technology.

The possible applications of selected cultures to technology might be very much further extended, but enough has been said to show the very wide field of work existing in this branch of bacteriology, and, whilst it is no exaggeration to say that modern bacteriology owes its origin to investigations connected with technical processes, and that, therefore, it is only meet and right that in an Institution devoted to the study of bacteriology, the industrial applications of this science should receive due recognition, yet, it cannot be denied that on its merits alone, and as a factor in the national welfare, technical bacteriology is worthy of every encouragement.

THE PATHOLOGY OF CANCER: AN EXPERIMENT.

BY H. LAMBERT LACK, M.D.

In March, 1898, I performed the following experiment at the Jenner Institute:—A healthy rabbit was anæsthetised, the peritoneal cavity was opened, the ovaries, which appeared to be healthy, were brought out into the wound, cut open, and the raw surfaces lightly scraped with the sharp edge of the knife. A milky juice was thus obtained, comparable to that seen on scraping the cut surface of a cancer, and this juice, rich in cells, was inserted into the peritoneal cavity. The operation was performed aseptically, the peritoneum was injured as little as possible, and the wound closed and dressed in the usual way. The animal soon recovered, and seemed well for about a year. Then it was noticed to become emaciated and weak, in spite of feeding as usual. Two months later it was much weaker, and began to suffer from dyspnœa. It was therefore killed.

On *post-mortem* examination the animal was found to be very emaciated. On opening the abdomen the liver was seen to be very large and hard, and to contain many little biliary cysts, and thickly scattered throughout it were numerous white hard nodules. These nodules varied in size, from tiny specks to the size of a cherry stone; in many places they coalesced, and especially along the margin of the liver they formed a continuous edging. Scattered throughout the mesentery were innumerable roundish nodules, all small and of cartilaginous consistence. The stomach, intestines, spleen, and kidneys seemed to be healthy. The ovaries had been scraped almost entirely away at the time of the operation, and no trace of them could be found. The uterus was thickened, its mucous membrane was papilliform, and in one part of its wall was a tumour about the size of a cherry.

The diaphragm was hard and thickened, and on opening the thorax large tumour-like masses were seen projecting from its upper surface. There was a chain of enlarged glands, extending along either side of the spinal column, large irregular tumours on the parietal surface of the pleura and in the upper lobes of the lungs. Many of these tumours were as large as horse beans, and all were gristly and hard on section. The pericardium was thickened throughout with similar tissue, and the whole mediastinal cavity was filled with one large mass of growth. Thus the thoracic cavity was involved to a far larger extent than the abdomen.

Pieces of the nodules in the liver, mediastinum, mesentery and uterus were prepared for microscopic examination. The tumour in the liver was found to consist of large irregular-shaped alveoli, lined by a single layer of columnar epithelium. The other tumours were in parts similar, but in other parts the alveoli seemed almost filled with epithelial cells of a more or less cubical shape. The microscopic characters of the growths were, as a matter of fact, similar to those of an ovarian cancer. The naked-eye appearances and the distribution of the growth showed that it had been conveyed by the lymphatic system—this being also typical of cancer.

Reviewing all the facts of this experiment, that the animal was affected with and was obviously dying from a disease having all the characters, macro- and microscopic, of ovarian cancer; and having regard to the distribution of the growth and the time of its appearance, there seems to me little doubt that the disease was the direct result of the setting free of the ovarian epithelial cells in the animal's peritoneum. Moreover, Professor Macfadyen, of the Royal Veterinary College, was kind enough to inform me that he had never seen or heard of cancer in rabbits.

Some three years ago I brought forward evidence to show that cancer was infective, and that the epithelial cells themselves were the infective agents.[1] As a matter

[1] *Lancet*, June, 1896.

of fact, the epithelium of a cancer does not differ essentially from the normal epithelium from which it arises. What, then, is a cancer? I believed the answer was to be found in the peculiar relation of the epithelium to the lymphatic system, that the cancer alveoli were simply dilated lymph spaces, and that cancer was due simply and entirely to the entrance of normal epithelium into the lymph spaces. I venture to believe that the above experiment shows that ovarian cancer can be produced by introducing free ovarian epithelium into the lymphatic system, but until the results of the further experiments I am conducting at the Institute are completed, it is well not to draw any general conclusions as to the cause of cancer. A fuller account of these considerations and experiments, with illustrations, appears in the *Journal of Pathology*, July, 1899.

ON NEISSER'S DIAGNOSTIC STAIN FOR THE DIPHTHERIA BACILLUS.

BY R. T. HEWLETT, M.D.

A NEW differential staining method for the diphtheria bacillus was recently published by Neisser.[1] The formula is as follows :—

1. One gramme of methylene blue (Grubler's) is dissolved in 20 c.c. of alcohol (96 per cent.) and mixed with 950 c.c. of distilled water and 50 c.c. of glacial acetic acid. 2. Two grammes of vesuvin are dissolved in 1000 c.c. of boiling distilled water, and the solution is cooled and filtered.

According to Neisser, cover-glass preparations are stained in No. 1 for one to three seconds, rinsed in water, counter-stained in No. 2 for three to five seconds, washed in water, dried, and mounted in Canada balsam. So treated the diphtheria bacillus appears as a slender, longish rod, stained brown and generally containing granules of a deep blue or inky tint.

There are usually two granules, situated at the poles, occasionally a third at the middle of the rod. The polar granules are ovoid rather than spherical in shape, and appear to be of a slightly greater diameter than the rod in which they are. In order to obtain these appearances the diphtheria bacillus must be cultivated on Loeffler's (ox) serum for not less than nine hours, and not more than twenty to twenty-four hours, preferably at a temperature of 34° to 35° C. Agar cultures hardly give the reaction at all. When these precautions are taken, few organisms react to the double stain like the diphtheria bacillus. The pseudo-diphtheria bacillus of Hoffmann morphologically differs entirely from it

[1] *Zeitschr. f. Hyg.* xxiv., 1897, p. 443.

and never shows any granules. With preparations of the xerosis bacillus, an individual here and there, resembles the diphtheria bacillus, but the majority do not react. Certain thread forms or "strepto-bacilli," such as the "leptothrix" in the mouth, show the double staining, but the threads are thick and the granules are large and spherical. The only other organisms out of a large number tried which gave the staining reaction were some vibrios (V. Nordhafen, Berolinensis and Finkler), and the plague bacillus.

I can confirm the striking staining reaction of the diphtheria bacillus by this method. It has been tested on about fifty cultures obtained from diphtheritic throats, and the characteristic appearance has been obtained in every case, and, with one exception, it was marked in all. The exception was an example of the short form of the Klebs-Loeffler bacillus and individuals showing the granules were very scanty; sub-cultures, however, reacted typically.

The pseudo-diphtheria bacillus of Hoffmann does not give the granulation. The staining solution employed has been prepared exactly according to Neisser's directions; it seems to keep well, for no difference was detected in the results given by ten months' old solutions and those freshly prepared. With regard to the times of staining, a slightly longer treatment than that recommended by Neisser has seemed to yield better results, viz., five seconds in the blue and ten seconds in the brown. After staining in the blue the specimen is rinsed in water, but any prolonged washing must be avoided.

Neisser lays some stress upon the fact that the polar granules are ovoid and not spherical in shape, but even with a $\frac{1}{20}$ in. oil immersion and careful illumination I have been unable to satisfy myself of this, so that it can hardly be made a diagnostic point under ordinary circumstances. A fairly high amplification (800 to 1,000 diameters) is necessary to obtain the full benefit of the method, and as the staining is somewhat faint it will generally be found advisable to diminish the light.

For the routine examination of the cultures for the

bacteriological diagnosis of diphtheria I think that Loeffler's blue is the preferable staining agent; it is simpler than Neisser's method, while the appearances obtained are more characteristic than by Gram's.

It is true that I had employed Loeffler's blue in many thousand cases before Neisser's method was devised and the personal element may therefore come in. Moreover, as I believe that Hoffmann's pseudo-bacillus is a modified Klebs-Loeffler, and that when it is present a positive diagnosis should be given, Loeffler's blue is an advantage, as it gives such a characteristic picture with this organism which the Neisser stain does not.

In doubtful cases, however, for example, when a bacillus is present which differs somewhat from a typical Klebs-Loeffler, or in very mixed cultures, Neisser's staining may be a very valuable confirmative method, and my experience leads to the conclusion that the reaction with Neisser's method is one of the most constant features of the diphtheria bacillus.

The difficulty often experienced in identifying directly the diphtheria bacillus in the membrane or swabbing from a throat led me to try the Neisser method. At first I had great hopes of it, but later experience seems to show that it is not infallible. The method adopted was to make a cover-glass specimen and stain in exactly the same way as a preparation from a culture. The preparation was then examined and a diagnosis made. At the same time a serum culture was prepared and examined next day in the ordinary way, thus controlling the Neisser method.

The following tables show the result obtained :—

I.—*Swabbings from throat; ? diphtheria. Material generally very scanty.*

	Neisser's method.	Serum culture.
1	+	+
2	O	O
3	O	+
4	O	O
5	O	Pseudo
6	O	O

	Neisser's method.	Serum culture.
7	O	O
8	O	O
9	O	O
10	+	O
11	O	O
12	O	+
13	O	O
14	O	O
15	+	+
16	+	+
17	+	O
18	+	+
19	+	O
20	+	+
21	+	+
22	O	+
23	O	O
24	+	+
25	O	+
26	O	+
27	O	O
28	+	O
29	O	+
30	+	O

\+ = Positive result as to presence of diphtheria bacillus.
O = Negative „ „ „ „ „

II.—*Membranes from ? diphtheritic throats.*

	Neisser's method.	Loeffler's blue.	Serum culture.
1	+	? v. mixed	+
2	+	„	+
3	+	+	+
4	O	O	+ (discrete colonies)
5	O	—	O
6	(? leptothrix filaments)	—	O
7	+	—	+
8	+	—	+
9	O	? v. mixed	O
10	O	„	O
11	+	+	+
12	O	?	O
13	O	?	+
14	O	?	+
15	+	+	+
16	+	+	+

For some of the membranes I am indebted to Dr. Kirton, of the Brook Hospital.

\+ = Positive result as to presence of diphtheria bacillus.
O = Negative „ „ „ „ „

Table I. (*swabbings*).—With regard to those cases in which the Neisser method gave a negative, and the serum cultures a positive, result, no stress need be laid, as it was hardly to be expected that a negative diagnosis could be established simply from a stained cover-glass specimen. But with regard to the positive results obtained, out of thirty cases an erroneous diagnosis was arrived at in five, judging by the serum culture controls.

In Cases 10 and 19 second swabbings were forwarded by the medical men in charge, but with the same result. Dr. Armstrong, of Newcastle, kindly furnished particulars with regard to Case 10; an antiseptic paint had been applied to the throat before the swabbing had been taken, which might have prevented a growth on serum, and it may therefore, after all, have been diphtheritic. Case 19, from particulars kindly forwarded by Dr. Mabyn Read, of Worcester, certainly did not seem to be one of diphtheria; with regard to Cases 17, 28, and 30 I have no information. This would give, therefore, an erroneous result in at least 14 per cent. of the cases.

It was also found that cover-glass specimens from the mouth of normal individuals, prepared by the Neisser method, frequently showed rod forms, which were indistinguishable from the diphtheria bacillus. In these cases, serum cultures gave negative results, so that the diphtheria bacillus was absent.

Table II. (*membranes*).—As will be seen from this table, the fresh membranes gave better results than swabbings with the Neisser method, and in many instances the appearances were most characteristic.

It is necessary, however, that the membranes should be fresh, and not treated with any preservative. Care must be taken that fragments of leptothrix (Neisser's "streptobacilli") be not mistaken for diphtheria bacilli. Micrococci in pairs, or diplococci and sarcinæ, also, sometimes simulate the diphtheria bacillus, the cocci being stained brown and having a dark granule in the centres, so that a pair resembles a diphtheria bacillus with polar staining. It is necessary, in order to be sure of the presence of diphtheria bacilli, to

exclude leptothrix forms, diplococci, dust, and *débris*, and to observe groups of bacilli of the characteristic size and shape, and reacting in the typical manner with the Neisser stain. With precaution to exclude fallacies, and using fresh membrane, Neisser's method will often afford a means of rapid diagnosis, superior to either Loeffler's blue or Gram's method. If a negative result be obtained, recourse must be had to culture methods, in order to establish the diagnosis.

AN INSTANCE OF SYMBIOTIC FERMENTATION.

BY ALLAN MACFADYEN, M.D.

THE study of the biology of fermentation has made great strides within recent years, and this has been notably due to modern bacteriological methods and the facilities they afford for obtaining pure cultures of the organisms to be met with in such natural processes. Investigation has shown that the simultaneous presence of two or more organisms in given processes of fermentation is a frequent occurrence, and that in many instances this constitutes an essential factor for their success. The net result is brought about by a co-operation in the activities of different groups of organisms. This co-operation, as has been found on closer study, may be of a symbiotic or a metabiotic nature. The antagonistic action of the micro-organisms is a fact that attracted earlier and wider recognition. We have, for example, the inimical action of the saprophytic on the parasitic forms of bacteria. The greater reproductive power, or the more favourable conditions of soil, may favour the development of one organism at the expense of another, or it may be that the products of one form are fatal to certain other forms of life. The water bacteria rapidly crowd out any extraneous forms that may gain access to water.

As has been indicated, actions of a co-operative nature on the part of micro-organisms are attracting the attention they deserve. The phenomena of putrefaction in their various phases are due not to the action of one species, but to the action of various species of aerobic and anaerobic bacteria. The complete study of the agents of such putre-

factive changes has yet to be accomplished, though their diversity is beyond question.

An organism by its growth may create the conditions that favour the development of another on the same soil. Thus in many morbid processes mixed and secondary infections are constantly noted. The infection of purulent wounds with the bacillus pyocyaneus was at one time a familiar example, whilst in the course of a disease such mixed infections may give rise to grave symptoms, and are marked features in diphtheria and tetanus. Infection is frequently aided by the injection of mixed cultures of organisms, *e.g.*, symptomatic anthrax with the bacillus prodigiosus, or an infection may be prevented by the same means, *e.g.*, anthrax by means of the bacillus pyocyaneus. These questions, however, lie beyond the limits of the present paper.

When one organism in the course of its growth renders a soil suitable for the subsequent development of a second organism, the process is termed metabiosis. A good example is to be found in the changes that occur in a natural wine must. An alcoholic fermentation of the fluid occurs in the first instance, resulting in the production of alcohol and CO_2. When the alcoholic ferments have finished their work new agents appear, and an acetic acid fermentation occurs which lasts until the alcohol has been used up. A third group of organisms now appear, which convert the acetic acid into carbonic acid and water. When this has been accomplished the changed soil becomes finally a prey to putrefactive bacteria, which had hitherto been unable to develop owing to the presence of the alcohol and acid.

It has been found that when two or more organisms are simultaneously inoculated in the same soil, a symbiosis may ensue whereby products are formed that do not occur when the organisms are cultivated individually. Nencki found that the bacillus of symptomatic anthrax when grown in grape sugar solutions forms H_2, CO_2, normal butyric acid and inactive lactic acid, whilst the micrococcus acidi paralactici forms almost entirely the optically active lactic acid. If, however, both organisms are cultivated together a more

active fermentation ensues whereby not only the above substances are formed but also a *new* product—normal butyl alcohol.

Neither the bacillus coli communis nor the bacillus denitrificans can liberate nitrogen from nitrates, but when cultivated together they produce a complete reduction of nitric acid to nitrogen (Burri and Stutzer).[1]

We have familiar examples of spontaneous symbiotic fermentations in koumiss and kephir. These alcoholic beverages are apparently due to the joint action of lactic acid bacteria and yeasts on milk. The "ginger-beer plant" is another instance that has been worked out with great care by Prof. Marshall Ward.[2] It may be as well to state that "ginger-beer plant" is a popular name in many parts of the country for a substance used in the home manufacture of ginger beer. Marshall Ward found that the "plant" contained a mixture of organisms amongst which were two essential forms, viz., a species of saccharomyces and a schizomycete—which he named respectively Saccharomyces pyroformis and Bacterium vermiforme. The former inverts cane sugar and produces an alcoholic fermentation in the inverted cane-sugar solution—the latter produces a lactic acid fermentation. It is only when these two organisms are present that a result similar to that of the ordinary ferment is produced; and further, they must be simultaneously present. Their relationship is that of a symbiosis and the result a typical example of a symbiotic fermentation. The yeast and bacterium are probably introduced attached to the ginger and brown sugar obtained from the grocers' shops.

Marshall Ward cites likewise the affection of the grapes in some Rhine districts known as "Edelfäule." "These grapes are rendered mouldy and 'rotten' by a species of botrytis which so alters the constitution of the grapes that the proportion of acids, sugar and nitrogenous matter are altered before they go into the must—such grapes yield wines of higher quality and finer 'bouquet' than merely ripe healthy ones similarly fermented."

Reynolds Green[3] refers to a parasitic growth on the

sugar cane which is composed of a yeast and a bacterium and produces a fermentation resulting in the formation of alcohol, CO_2, acetic and succinic acid.

The action of mixed cultures of yeasts is of great importance in brewing operations. "Many of the best brews are known to be due to yeasts which are not of a pure strain in Hansen's sense, and it is not at all improbable that a better brew can be obtained by symbiotic ferments than by a pure one."[2]

As I have indicated, the whole subject is attracting increased attention. The large processes of nature, such as the re-distribution of cellulose and other forms of dead matter, are due to the co-operation of many living agents. This fruitful subject of inquiry is being gradually opened out, and results of great scientific and practical value may be anticipated. The following account does not lay claim to originality, as the results are simply a control of those obtained by others. They may perhaps serve to draw further attention to this promising line of investigation, dealing as they do with the successful applications of a pure symbiotic fermentation.

The production of alcoholic beverages, such as wine, beer, and spirits, is due to the action of living agents or "ferments" on sugars. The vegetable cells or yeasts employed for this purpose split up the sugar into alcohol and carbonic acid. The sugar may exist formed as in the case of the manufacture of wine from the grape, or it may have to be prepared as in the ordinary distilling operations. In the latter instance the sugar is generally prepared from starch by the addition of malt, which, in virtue of a saccharifying ferment it contains, dissolves and converts the starch in the grain employed into a fermentable sugar. To this sugary fluid the yeast is added, and an alcoholic fermentation ensues. We have, therefore, a twofold action :— (1) the saccharifying action by the malt ferment resulting in the production of sugar from starch ; (2) the ferment action by the yeast resulting in the production of alcohol from the sugar. The success of such processes depends upon the use of the right agents. The yeasts are a widely

distributed group of micro-organisms, and consist of a great variety of species. Of these, some species are specially adapted for employment in industrial fermentation processes, others are not. It is necessary to have the right kind of yeast to ensure a successful result. The technical advance made in bacteriological methods has enabled brewing chemists to cultivate the useful yeasts on suitable soils, and to conduct their processes with pure breeds of the right sort. In this respect the labours of Pasteur and Hansen led the way to the substitution of scientific for rule-of-thumb methods of work.

The alcoholic fermentation is a biological process, and an acquaintance with and a supervision of the living agents to which it is due are essential factors in its successful manipulation. The importance of this fact is now recognised by the establishment of zymotechnical laboratories for the determination of the organisms that can be most advantageously employed, and for the cultivation of the same for use in brewing and allied processes. This has led to a more accurate adaptation of the means to the end in view. But the selection of the right agents is not the only matter of importance ; care must also be taken to prevent the action of injurious organisms during a fermentation process. These, if present, will develop along with the essential factors in the fermentation and interfere with the yield and quality of the alcohol. This is one of the great troubles in connection with fermentation industries of the above nature, and has been partially combated by strict attention to cleanliness in all the operations with a view to preventing the intrusion of microparasites, which either hinder the fermentation or act injuriously on its products. The ubiquitous nature of the organisms in question renders this latter point a matter of great difficulty, and the attempt has been made to overcome it by the addition of antiseptics to the wort. The absolutely certain method of killing such infective agents by heat is impossible in the ordinary methods of distillation, inasmuch as the temperature necessary to effect this would destroy the saccharifying action of the malt upon the starch. An element of uncertainty as re-

gards results, therefore, remains in the distilling processes usually employed.

In the *Annales of the Pasteur Institute* M. Calmette[4] published a paper upon "La Levure Chinoise," or the Chinese Yeast. As already stated, the first step in the manufacture of alcohol in European countries is the conversion of starch into sugar by the action of an unorganised ferment, the malt diastase, or sometimes by the action of acids. In Eastern countries, however, organised or living ferments have long been used to saccharify the starch, *e.g.*, the Japanese Koji, which is used to prepare an alcoholic beverage from rice, called Sake. The agent in this instance is a mould, the *aspergillus orizæ*, and the saccharified fluid subsequently undergoes a spontaneous alcoholic fermentation in which yeasts take part. The Koji is also used by the Japanese for the preparation of the sauce "Soy" and in the making of bread. Takamine has devoted special attention to the study of this ferment and the utilisation of pure cultures of the mould as saccharifying agents.[5] Similarly in China and Indo-China a special ferment has been used, which M. Calmette found to be distinct from the Japanese Koji. This "Chinese Yeast" consists in the symbiosis of a mould and several varieties of alcoholic yeasts. It appears in commerce as flattened cakes. The recipe for its preparation includes about forty-six aromatic ingredients, but no mention is made of the living agents which were introduced into the cake with the rice grains used in its manufacture. The Chinese were, therefore, apparently ignorant of the essential factor in their process. As can be readily understood, such a mixture contained a number of impurities, which could only act deleteriously on the process, as the smell of a sample of the native spirit sufficiently testified. The Chinese add this "yeast" to a rice rich in starch, called "Nep." A saccharification of the starch occurs in three days and an alcoholic fermentation in two days. The liquid is then distilled in a primitive form of apparatus.

M. Calmette succeeded in isolating the essential factor from the crude "Chinese Yeast" by means of bacteriological methods, using a solid nutrient soil for this purpose, viz.,

beerwort gelatin. Growths were obtained of several yeasts and several species of moulds; one species of mould was particularly numerous, and its branching filaments rapidly covered the whole surface of the nutrient soil employed. This mould was also easily cultivated on other soils, such as potato, beerwort, rice, grains, &c. It also actively hydrolysed starchy media, producing dextrin and fermentable sugars. In this respect it was the most energetic fermentative constituent of the Chinese yeast, and undoubtedly the main factor in its action. The mould we are dealing with belongs to the group of the mucors, and was named by M. Calmette the *amylomyces rouxii*. The habitat of this mould appears to be the undecorticated rice grains, from which it can be readily isolated. The reproduction of the mould is brought about by means of spores, which are formed in special spore-sacs if the organism has free access to air, or they are formed in the continuity of its filaments if the access of air is limited. These spores preserve their vitality for a considerable time (two to three years), and if transplanted to a fresh soil reproduce the characteristic growth of the mould once more. There is little else to be seen in old cultures of the mould save the reproductive spores, and it is these spores which are utilised when it is desired to start a new growth and to set the special functions associated with the vegetative life of the mould at work. The useful functions of this mould vary with the amount of air to which it has access. If the amount of air be limited, or if growth takes place below the surface of a sugary fluid, the fermentative power of the organism is increased. The Chinese recognise this in a primitive fashion by placing a cover on their fermentation jars. A second point of practical importance is the temperature at which the mould is cultivated. The development and saccharifying action of the amylomyces rouxii occur best at 35° to 38° C.—*i.e.*, at temperatures approaching blood heat. The experiments of M. Calmette have thus shown that the "Chinese Yeast" contains a special ferment, and consists in the symbiosis of a saccharifying mould (amylomyces rouxii) with one or more wild alcoholic yeasts.

The amylomyces rouxii can also ferment the sugar it has formed, but experience has shown that the process works better when the action of a yeast is called in as well.

M. Sanguinetti[6] conducted a series of comparative experiments in M. Calmette's laboratory upon certain moulds which have the double property of fermenting starch and sugar, viz., the mucor alternans (Gayon), the aspergillus orizae (Japanese Sake ferment), and amylomyces rouxii (the Chinese ferment). It was found that the amylomyces rouxii was the best adapted for industrial use on account of its marked saccharifying and fermenting properties, as well as from the fact that its oxidising properties are feeble, and, consequently, it does not produce loss by the destruction of valuable products of fermentation.

Upon these observations of M. Calmette a process of distillation has been founded, which is far removed from the primitive methods employed in the East. Biologically the process consists in sowing the spores of amylomyces rouxii in a sterilised mash, allowing the organism to develop at a suitable temperature to saccharify the mass, and then adding a pure culture of a yeast to produce a complete alcoholic fermentation of the sugar so formed—the whole process being carried out under aseptic conditions in sterilised chambers or vats. The mash cannot be sterilised in the ordinary processes of fermentation, because the malt diastase which is used for saccharification is destroyed at temperatures approaching the boiling point. In this method complete sterilisation is possible, the saccharification being accomplished by the amylomyces rouxii that is added subsequent to the sterilisation of the mash. The conditions are entirely aseptic throughout, and represent the careful methods of the bacteriological laboratory carried out on a large scale. I have to thank M. Calmette for a pure culture of the amylomyces rouxii.

The organism is readily cultivated on the ordinary culture media of the bacteriological laboratory. It gives a good growth on beerwort agar at 22° C. and at 35° to 38° C. At the higher temperatures its development is particularly vigorous, and the whole surface of the medium

employed becomes covered with a silky growth in the space of twenty-four hours. Sown in liquid beerwort the growth is of an equally vigorous character. In sugar agar the organism also develops well, and the growth is accompanied by the development of gas bubbles, due to a fermentation of the sugar present. There is also a development of the "amylo" in the ordinary bouillon made from beef, and a felt-like growth occurs on the surface of slices of potato. On nutrient gelatin the "amylo" growth is accompanied by a liquefaction of the medium. In simple starch water the growth permeates the whole of the fluid in twenty-four hours.

When sown on slightly moistened rice grains the development is slower than on the above media. The growth when it does appear in 2 to 4 days consists of delicate aërial filaments or hyphæ, which spin themselves in a gossamer-like fashion round each individual rice grain. The sporulation of the organism on the rice grains proceeds quickly, and as the culture becomes older there is little else but these reproductive elements or spores to be seen. This soil is the best for obtaining stock cultures of the amylomyces rouxii, because the fructification that occurs is so complete, and the spores thus formed preserve their vitality for a long time. Such a culture can be used after many months for starting a fresh growth when required.

The following experiment, which can be readily carried out, is a happy illustration of a pure symbiotic fermentation: If the amylomyces rouxii be grown on boiled and moistened starch grains for about eight to ten days at a temperature of 33° C., a distinct, visible web-like growth appears on the surface of the individual rice grains. If this culture be added to sterilised distillers' wash, and the fluid kept at blood heat with free access of air, a very active growth occurs, and the whole liquid becomes filled with the thread-like filaments of the organism. The result is an active saccharification of the starchy material. If at an interval of about twenty-four hours a pure culture of a yeast be added to the flask, it undergoes a rapid development alongside of the mould in the now saccharine fluid. The yeast's growth appears to be

favoured and accelerated in the fluid which is interpenetrated with the filaments of the mould. If we may so express it, the two organisms join hands, the mould saccharifying the starch, and the yeast fermenting the sugar as quickly as it is formed into alcohol and carbonic acid. This symbiosis accomplishes more than if either organism were working alone, and at the same time their respective action is unhindered by foreign organisms, as is the case in the crude Chinese process of fermentation. The following experiments, suggested by Dr. Horace Brown, will serve to illustrate the results of the symbiosis I have endeavoured to describe. I am indebted to him and to M. Boidin for the analytical data that are given below.

A series of flasks are prepared containing well sterilised wash, and after treating the flasks in the manner described below, they are placed at a temperature of 32° C. for five days :—

Flask 1A „ 1B	Seeded with yeast only.
„ 2A „ 2B	Seeded with "amylo" only.
„ 3A „ 3B	Seeded both with "amylo" and yeast, which were *added simultaneously*.
„ 4A „ 4B	Seeded first with "amylo" and then, *twenty hours afterwards*, with yeast.

At the end of the five days the appearance of the flasks differed very much.

In 2A and 2B the growth of the "amylo" covered the entire surface of the liquid. In 3A and 3B, which had been seeded simultaneously with both yeast and "amylo," only a few small isolated patches of "amylo" appeared on the surface. The growth of "amylo" was meagre, the carbonic acid produced by the yeast having interfered with its development. In 4A and 4B, where the seeding with "amylo" was followed by the addition of the yeast twenty hours afterwards, there was a much larger growth of the "amylo" film.

The results of the chemical examination showing the extent of fermentation in each case are here tabulated :—

	Yeast Only.			"Amylo" Only.	
	Control.	1 A	1 B	A	2 B
Total acidity	0·029	0·068	0·068	0·147	0·156
Fixed acidity	0·029	Lost	0·044	0·102	0·110
Iodine reaction	Violet	—	Violet	None	None
Alcohol per cent. weight	0	—	2·189	2·620	2·519
Apparent density	1031·3	—	1008·9	1008·8	1010·0
Real density after separation of alcohol	—	—	1013·1	1013·7	1013·9

	"Amylo" and Yeast.		"Amylo" with Yeast after Twenty Hours.	
	3 A	3 B	4 A	4 B
Total acidity	—	0·068	0·127	—
Fixed acidity	—	0·058	0·080	—
Iodine reaction	—	None	None	—
Alcohol per cent. weight	3·16	3·920	4·135	4·085
Apparent density	1007	1006·0	999·4	999·6
Real density after separation of alcohol	1011·3	1010·6	1005	1005·4

It will be seen from the above experiments that the mould when grown alone can both saccharify starch and ferment the sugar formed, but that the combined action of the mould and yeast is greater than the action of either organism when grown alone. This symbiotic action is more active if the yeast is added subsequently to the mould.

These experiments illustrate facts of considerable biological interest, viz. :—

(1) The utilisation of pure cultures of a mould instead of diastase for purposes of saccharification and fermentation.

(2) The aseptic conditions that this procedure renders possible.

(3) The development of symbiotic activity that occurs when the mould and a yeast are cultivated together in an originally sterile fluid, the result being a pure symbiotic fermentation.

It cannot, of course, be supposed that these properties are peculiar to the amylomyces rouxii, and it remains for future research to determine in how far other species of moulds may be adapted for utilisation in a similar fashion.

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ON SOME ORGANISMS OF THE BACILLUS COLI COMMUNIS GROUP ISOLATED FROM DRINKING-WATER, &c.

BY JOSEPH LUNT, B.Sc.

THE bacillus coli communis has been invested with so much importance as an indication of sewage pollution, when met with in water, that it has become customary, in carrying out a bacteriological examination of water, to make a special search for this organism.

During the course of the examination of many waters at the Jenner Institute this organism, and others closely resembling it, have frequently been met with, and in a large number of cases the organisms have been examined in various ways to ascertain whether they give all the test reactions which a normal variety of bacillus coli communis will answer to.

Those who assign such importance to the presence of bacillus coli communis in water, have to consider what tests they will regard as essential in judging whether an organism is bacillus coli communis or not, and whether when an organism is found which in one or more respects differs from the normal variety it should be assigned the same importance as an evidence of pollution, in spite of its peculiarities.

An examination of the organisms here described shows that there is scarcely one of the many tests for bacillus coli communis which does not break down completely, in the case of one or more of these allied organisms, although they may be perfectly normal in many respects.

The indol reaction, the curdling of milk, the motility of the organism, the absence of liquefying power, the reaction with sugar-litmus agar, the appearance of the colonies;

none of these can be regarded as infallible tests if we regard all the closely allied organisms as mere variations of bacillus coli communis having equal importance as evidence of the sewage pollution of water.

Some experimenters are in favour of condemning all waters which contain "pseudo"-typhoid bacilli, but some of the organisms described in the present paper are intermediate in character between bacillus coli communis and bacillus typhi abdominalis, possessing some of the characters of both, and may equally well be named "pseudo"-coli, or "pseudo"-typhoid bacilli.

It is quite probable that the bacillus of typhoid may be subject, under certain conditions, to similar variations to those shown by bacillus coli communis, and thus the group of "pseudo"-coli or "pseudo"-typhoid acquire great importance for the water bacteriologist.

A case in point may be cited. On making a bacteriological examination of the urine af a typhoid patient, mainly normal bacillus coli communis was met with, but one culture had two of the characters of the typhoid bacillus most strongly marked in that the bacilli were exceedingly motile, resembling the typhoid bacillus in this respect exactly, and the cultures gave no trace of indol reaction when grown side by side with the normal coli, which under identical conditions gave a most pronounced indol reaction. This organism agreed with bacillus coli communis in curdling milk in twenty-four hours, and in giving off gas in melted and resolidified gelatine, and in sugar-litmus agar, which latter was rendered acid and remained so.

In another case an organism isolated from the water supply of a large town resembled the typhoid bacillus in every respect, except that it was non-motile, whereas the normal bacilli show vigorous motility, and in older cultures a good indol reaction was obtained, whereas the typhoid bacillus gives none.

It was very preplexing to decide what importance to ascribe to the presence of this organism, which could not be said to be the typical typhoid bacillus and which yet resembled it so closely; and other considerations had to be

relied on in forming an opinion on the potability of the water, which was not condemned.

Then, again, an organism was frequently met with in samples of good water, which resembles bacillus coli communis in every respect except that it does not curdle milk. Another variety has been met with which not only does not curdle milk but also fails to give the indol reaction, in these respects agreeing with the typhoid bacillus whilst in all other respects it agreed with bacillus coli communis.

It is worthy of notice that this latter organism was isolated from a sample of well-water which had been sent to the water laboratory at the Institute for examination on suspicion of being concerned in an outbreak of typhoid fever; and the chemical and bacteriological examination resulted in a condemnation of the water for drinking purposes, apart from the presence of this "pseudo"-coli or "pseudo"-typhoid bacillus.

The above variety was found to show incipient liquefaction after seven days and the gelatine became liquefied on the tenth day, although in the earlier days the culture both in gelatine plate and on sloped gelatine had been quite indistinguishable from the normal bacillus coli communis.

Another variety was met with in the same water which could only be distinguished from normal coli in that it gave no indol reaction and liquefied slowly after thirteen days.

These characters were not determined by a single examination. In all cases the cultures were examined over and over again, and found to persist in their peculiarities, and not only so, but they were examined side by side under the same conditions repeatedly.

Especially is this important in carrying out the indol reaction; a normal coli was always grown side by side with those which gave no indol reaction, and for such lengths of time (nine days and more) that a very pronounced reaction was given with the normal coli.

An interesting observation was made during the examination of a number of cultures of bacillus coli communis isolated from sewage-polluted oysters.

A series of indol reactions had been tried, and half the

broths had been sown from sloped gelatine cultures grown at 23° C, the other half from sugar-litmus agar cultures grown at 37° C. In almost every case the indol reaction obtained in the broths sown from sugar-litmus agar was decidedly more pronounced than that obtained in the broths sown from the gelatine culture, so much so that it was possible to pick out from the series those cultures sown from each medium without referring to the labels. The trace of culture used for sowing the broths from sugar-litmus agar was too minute to cause any suspicion of litmus being concerned in the result above noted.

In the case of milks not curdling, they were in all cases sub-cultured after several days to make perfectly sure that the sowing had been effectual, and a normal coli giving a curdling in twenty-four hours was grown side by side with them in the same batch of sterile milk tubes.

In short, every precaution was taken which would exclude experimental error.

The curdling reaction with milk seems to be the most variable character. Some of the organisms which at first gave a negative reaction ultimately in older cultures gave a positive one, and the conditions under which the milk was sterilised seemed to affect this.

The above examples render it clear that organisms may be, and are, frequently met with, which have a very close resemblance to the typhoid bacillus or the bacillus coli communis, and many of which may be regarded as intermediate in character between the two organisms.

It is also clear that it is easy, by an incomplete bacteriological examination of the characters of an organism, to imagine that one has isolated the typhoid bacillus from water. When such is not the case, and when this happens to occur with a polluted water, which is, at the same time, under suspicion of being concerned in an outbreak of typhoid fever, such a finding of a spurious typhoid bacillus would be recorded as an authentic instance in which the typhoid bacillus had been isolated from water.

These results indicate that little reliance can be placed on many of the earlier reported instances of the finding of

the typhoid bacillus in water. Perhaps the only test which promises to be at all decisive is the serum reaction, but the application of this test is not within the reach of all who carry out bacteriological examinations of water. The highest expert evidence is, in such instances, undoubtedly a *sine qua non*, when dealing with reported evidence of the presence of the typhoid bacillus in a sample of water, and in many cases this has been lacking.

The number and character of the flagella, also, cannot be relied on, as has been proposed, to distinguish between the true typhoid bacillus and its "pseudo" forms.

So far as the author's experience goes, although he has examined a very large number of cultures of bacillus coli communis obtained directly from normal fæcal matter, he has never yet obtained an undoubted cultivation of this organism from fæces which was abnormal in any respect.

Moreover, with a view to test whether the normal organism becomes abnormal after prolonged residence in water, water cultures were made.[1] These showed absolutely *no* multiplication in this medium, a great contrast in this respect to water-bacteria proper; indeed, they showed, after five months, a marked decline in numbers, *but the survivors retained their normal characteristics entirely* unimpaired. Six colonies from plate cultures were examined and they all curdled milk in twenty-four hours, gave a pronounced indol reaction, acidified sugar-litmus agar with production of gas, gave an evolution of gas in melted and resolidified gelatine, and were practically non-motile.

Evidently, then, the mere existence in water for five months had no effect whatever in modifying the characters of normal bacillus coli communis from fæcal matter in the direction of the organisms here described, and it becomes an interesting point to ascertain the conditions under which a normal bacillus coli communis may be so modified as to agree with those found in water.

I now proceed to give a description of the normal colon bacillus and its allied forms, which have been isolated by

[1] Lunt. On a convenient method of preserving living pure cultivations of water-bacteria. *Trans. Brit. Inst. Prev. Med.*, First Series.

me from various sources. In such investigations it is important that the culture media are prepared according to uniform and standard methods, as variations in alkalinity, &c., have an influence on the growths obtained.

I. *Bacillus Coli Communis (Normal).*

Where found.—In fæces, in sewage, in sewage-polluted oysters, and in polluted water—once in a town's supply of excellent quality, but most probably this was a local contamination at the point of collection, the sample having been collected at a hydrant close to a cab-stand strewn with horse manure.

How isolated.—Both by direct plate cultivation from the water, &c., and by plate cultivations from carbolised broths sown with the water and incubated for twenty-four to forty-eight hours at 37° C.

Gelatine plates.—The colonies appear in twenty-four hours, those in the depth as small, white dot colonies, those on the surface having attained a diameter of about 2 mm.; they appear as thin, iridescent expansions of circular outline and with crenated and irregular edges. On the second day the surface colonies attain a diameter of 5 to 6 mm. and form thin, grey, translucent expansions, which under a low power appear to be pencilled over with fine markings in various directions; the colony is well defined, almost circular, but with thin, crenated edges.

Gelatine streaks grow rapidly. In twenty-four hours the growth is often 4 to 5 mm. in diameter, and in character resembles the colonies. No liquefaction ever takes place even in old cultures.

Gelatine stabs.—The path of the needle in the depths is much thickened and on the surface the same thin, translucent expansion appears as in colonies on plates.

Melted and resolidified gelatine.—In twenty-four hours the gelatine is riddled with bubbles of gas and the gelatine is rendered turbid.

Broth becomes very turbid in twenty-four hours at 37° C. and only a very faint film is formed on the surface. The cultures have a distinct odour.

Agar agar.—A thin, moist, translucent, grey expansion covers about half the surface of the tube.

Sugar-litmus agar.—The medium is turned red in twenty-four hours and the depth of the agar contains numerous gas bubbles, which are also to be seen on the surface of the exuded liquid. The surface growth becomes tinged slightly with the reddened litmus.

Potato.—A thick, moist, yellowish-grey expansion forms at 37° C. on the inoculated surface, which becomes brownish in old cultures. At other times the growth may resemble that given by typhoid.

Litmus milk curdled in twenty-four to forty-eight hours. The whole of the milk is turned into a solid coagulum and a watery fluid separates on inclining the tube, the litmus is reddened and frequently bleached completely in the depth.

Peptone water at 37° *C.*—Not so favourable a medium as broth; still, the organism grows well and gives the indol reaction in twenty-four hours.

Indol reaction.—Both broth and peptone water cultures forty-eight hours old at 37° turn red on the addition of 1 c. c. of a 0·02 per cent. solution of potassium nitrite and 0·5 c.c. of sulphuric acid.

Optimum temperature, 37° *C.*, grows well on all media at 37°; grows rapidly on gelatine at 23° C.

Effect of Parietti's solution, grows well in 10 c.c. of broth to which has been added 0·3 c.c. of Parietti's solution. This seems to be about the maximum.

Oxygen requirements, will grow well in pure hydrogen or nitrogen and is a facultative anærobe.

Microscopic preparations.—Cover-glass impressions show at the edges of the colonies short rods with rounded ends, usually single, but occasional threads are visible. The uppermost layers at the centre of the colonies appear as thinner and much shorter rods, often almost cocci. These are the oldest bacilli of the colony and are raised up out of contact with the food material by the growth of the deeper layers and evidently assume a resting stage. The microscopical appearance of these coccus forms is strikingly distinct from the vigorous rods at the thin margin of a

rapidly growing colony. In twenty-four hours broth cultures the—

Motility is not very marked, the larger proportion of the rods being motionless, but occasional vigorous motility is observed in some individuals.

Spore formation not observed.

Staining reactions: Stains well with methyl violet.

II. *Bacillus Coli Communis. Variety A. (Not curdling milk.)*

Where found.—In many potable waters, good and doubtful samples alike containing it.

How isolated.—From plate-cultures made from carbolised broths sown with the water and incubated at 37° C. for 24 to 48 hours.

General characters.—This organism is quite indistinguishable from normal bacillus coli communis in all respects except that it does not produce curdling in milk at 37° C. even when incubated for several days (7 to 10 days). The organism can be found in the non-curdled milk after incubation. It gives off gas in melted and resolidified gelatine and in sugar-litmus agar, which is turned acid in twenty-four hours; it gives a good indol reaction, thus distinguishing it from the following variety (B).

In motility and in its behaviour with carbolised broth at 37° C. it agrees with normal bacillus coli communis.

III. *Bacillus Coli Communis. Variety B. (Not curdling milk; no indol.)*

Where found.—In many potable waters, good and doubtful samples alike containing it.

How isolated.—From plate-cultures made from carbolised broths, sown with the water and incubated at 37° for twenty-four hours.

General characters.—The only points in which this organism differs from normal coli are that it does not curdle milk, nor give a decided indol reaction. It gives off bubbles of gas in melted and resolidified gelatine and in sugar-litmus

agar, which is reddened in twenty-four hours. After repeated sub-culture its fermentative action on sugar-litmus agar becomes less pronounced, and, although rendering the medium acid and reddening the litmus, it sometimes fails to produce any bubbles of gas in that medium.

In gelatine plate and streak cultures it is quite indistinguishable from normal bacillus coli communis.

This organism is distinguished from the last variety (A) in giving no indol reaction, but it agrees with it in not curdling milk.

IV. *Bacillus Coli Communis. Variety C.* (*Not curdling milk; no indol; liquefying in old cultures.*)

Where found.—In a polluted well-water suspected of being concerned in an outbreak of typhoid fever.

How isolated.—By plate-culture from a carbolised broth tube sown with the water and incubated at 37° C. for twenty-four to forty-eight hours.

General characters.—This organism agrees with the last in giving no indol reaction and in not curdling milk, but it has the added peculiarity of causing liquefaction of the gelatine in sloped cultures after seven to ten days.

V. *Bacillus Coli Communis. Variety D.* (*Marked motility; no indol.*)

Where found.—In the urine of a typhoid patient.

How isolated.—By plate cultivation from a carbolised broth tube. In the plate-culture it occurred side by side with the normal bacillus coli communis.

General characters.—All the cultural characteristics are the same as the normal coli. It gives off bubbles of gas both in melted and resolidified gelatine, and in sugar-litmus agar, which is reddened in twenty-four hours. Milk is curdled in twenty-four hours. The points of distinction between it and the normal coli are the very marked motility of the bacilli in broth cultures and the inability to give an indol reaction. In these two respects, therefore, this organism agrees precisely with the typhoid bacillus, but in all other respects it agrees with bacillus coli communis.

After repeated sub-culture it retains these characteristics. A broth culture seven days old shows marked motility, whereas the normal colon bacillus shows no motility. The same seven days old culture shows no indol reaction, whereas bacillus coli under the same conditions gives a pronounced indol reaction.

VI. *Bacillus Coli Communis. Variety E.* (*No indol; liquefies gelatine after ten to thirteen days.*)

Where found.—In a polluted well-water, which was suspected of being concerned in an outbreak of typhoid fever.

How isolated.—By plate-culture from carbolised broth tubes sown with the water and incubated at 37° C. for twenty-four to forty-eight hours.

General characters.—This organism agrees with bacillus coli communis in plate-culture and gelatine streak-culture. It, however, gives no indol reaction, and after ten to thirteen days begins to liquefy the gelatine, which in older cultures becomes completely liquefied. It curdles milk in twenty-four hours, gives off gas in melted and resolidified gelatine, although this reaction sometimes fails. In sugar-litmus agar it also gives off gas and reddens the medium. The acidity which first appears is succeeded by alkalinity in three to six days, and the medium is turned blue.

VII. *Bacillus Coli Communis. Variety F.* (*Liquefies gelatine after seven to ten days; no indol; curdles milk after three to five days.*)

Where found.—In a potable water of fair quality.

How isolated.—Isolated by plate cultivation direct.

General characters.—The plate cultures very closely resemble normal coli; will grow in 0·03 per cent. carbolised broth; gives evolution of gas in melted and resolidified gelatine, and also in sugar-litmus agar, which is first acidified, then bleached, and on the second day rendered *alkaline;* actively motile in broth at 37° after twenty-four hours' growth. The cultures begin to liquefy after seven to ten

days, and ultimately the whole of the gelatine in a streak-culture becomes liquid.

Plate-cultures (gelatine).—Resembles normal coli very closely; growth is, however, somewhat thicker, more opaque, and whiter than normal coli, yet falling within the limits of variation. Rate of growth is exactly the same as normal coli.

Gelatine streaks.—Almost indistinguishable from bacillus coli for the first few days. After seven to ten days, however, the gelatine becomes softened down the centre of the streak-culture, which soon afterwards slides down in the middle, and ultimately the whole of the gelatine becomes liquid.

Melted and resolidified gelatine.—Good evolution of gas in twenty-four hours, the medium becomes riddled with bubbles.

Sugar-litmus agar.—In twenty-four hours the medium is reddened, and a copious evolution of gas takes place. Sometimes after twenty-four hours a very marked bleaching action takes place, the colour being almost completely discharged; the agar then appears of the usual yellowish colour. The action of the air restores the colour by oxidation on the second or third day, when the medium is coloured blue owing to its alkalinity. The alkaline reaction then persists, even in old cultures. At the bleaching stage the reaction of the exuded fluid at the bottom of the tube in which the bacilli have grown is markedly alkaline to litmus paper.

The growth on this medium is markedly thicker, whiter, and more opaque than that of normal coli.

Milk.—Not curdled in twenty-four hours. After three to five days curdling takes place, and sometimes bubbles of gas collect in the coagulum.

Effect of Parietti's solution.—The bacilli grow in 0·3 per cent. carbolised broth.

Motility.—In a twenty-four hours broth culture grown at 37° active motility is observed, and there is a greater tendency to form threads than is the case with normal coli. When taken from a gelatine-plate culture, and suspended in water, no motility is, however, observed.

Indol reaction.—Broth cultures give no indol reaction.

VIII. *Bacillus Coli Communis. Variety G.* (*Liquefies gelatine after five to nine days; no indol; milk not curdled.*)

Where found.—In potable water of very good quality.

How isolated.—By direct plate cultivation.

General characters.—Plate-cultures very closely resemble normal bacillus coli for which it may be very easily mistaken. Plates grown side by side with normal coli are quite indistinguishable. Agrees with the typhoid bacillus in acidifying sugar-litmus agar without the production of gas; no evolution of gas in melted and resolidified gelatine; no indol reaction; milk not curdled, and is non-motile. It liquefies gelatine slowly after five to nine days, before which time its resemblance to bacillus coli communis (normal) is complete.

Gelatine plates.—Quite indistinguishable from normal bacillus coli communis, both in appearance of colonies and rate of growth.

Gelatine streaks.—Quite indistinguishable from normal bacillus coli for the first few days. After five to nine days it begins to soften the gelatine in the middle of the streak, and the central portion of the streak slides down and ultimately the gelatine becomes completely liquefied.

Melted and resolidified gelatine.—When first isolated it produced gas bubbles in gelatine in twenty-four hours, but after repeated cultivation it lost this power.

Sugar-litmus agar.—Grows exactly like typhoid bacillus; the medium is acidified and reddened in twenty-four hours without the evolution of gas bubbles. The appearance of the growth is exactly like that of the typhoid bacillus. The growth never becomes alkaline, like variety F.

Milk.—This organism is unable to curdle milk.

Indol reaction.—Broth cultures give no indol reaction.

Motility.—The rods are non-motile and exhibit a tendency to form long threads.

IX. *Bacillus Coli Communis. Variety H.* (*Non-motile; positive indol reaction.*)

Where found.—In a town's water supply of fair quality, no typhoid outbreak present. (Number of organisms per c.c. of the water, 140.)

How isolated.—Isolated by plate-culture from carbolised broth (0·02 per cent.) infected with the water.

General characters.—In cultures this organism most closely resembles the typhoid bacillus; it withstands 0·2 c.c. of Parietti's solution, grows exactly like the typhoid bacillus in gelatine cultures; gives off no gas in melted and resolidified gelatine; acidifies sugar-litmus agar without the evolution of gas, and gives no indol reaction in forty-eight hours old broth culture, although in older cultures the indol reaction is observed.

In one important respect, however, it differs from the typhoid bacillus; *i.e.* the rods are non-motile, whilst the typhoid bacillus is very actively motile. Injected intra-peritoneally into guinea-pigs, it caused death in twenty hours, and the bacillus was again isolated from the peritoneal exudation by first sowing into carbolised broths, and afterwards making plate cultures from the turbid broths which had been incubated for two days at 37° C. It retained the above peculiarities.

The above list of organisms does not, of course, exhaust the number of possible forms that may occur in water, and which may give rise to confusion by resemblances to the typhoid bacillus or the typical colon bacillus. The colon bacillus has hitherto been the sheet-anchor of many bacteriologists, as a biological test of sewage contamination, and certainly this ubiquitous organism ought not to be present in any number in a sample of water. The above observations will illustrate the care that is necessary for absolutely proving its presence. The task has not been rendered easier by the published results of other observers. Dr. Gordon (*Journal of Pathology and Bacteriology*, June, 1897) isolated at least sixteen varieties of colon-like organisms from the dejecta, sewage, water and milk. With regard to the detection of the typhoid bacillus the serum test would appear to be indispensable.

ACTION OF HYDROGEN PEROXIDE AND THE OXIDES OF COPPER ON FORMALDEHYDE.

BY ARTHUR HARDEN, M.SC.

FORMALDEHYDE is one of the most efficient preservatives of food which is known to us, and is on this account largely used for the preservation of such materials as milk, butter, &c. Under these circumstances, it is of great importance that an accurate and convenient method should be found for estimating the amount of formaldehyde present, both in commercial formalin, and in the more dilute solutions which are sold and largely used for the preservation of milk. Although the experiments recorded in the present paper have not yet led to the solution of these problems, they have brought to light certain reactions of the aldehyde, upon which it is hoped that a method of quantitative estimation may be based. The experiments which are being made in this direction are, however, not yet complete.

When a solution of formaldehyde is mixed with hydrogen peroxide no action occurs, but when the liquid is made strongly alkaline with caustic soda, a considerable amount of heat is developed and a vigorous effervescence occurs. The gas evolved consists of hydrogen, which is practically pure and free from carbon monoxide, carbon dioxide and oxygen.

Quantitative experiments show that the reaction proceeds according to the equation—

$$2\,CH_2O + 2\,NaOH + H_2O_2 = H_2 + 2\,H.CO_2Na + 2\,H_2O.$$

In order to ascertain these quantitative relations a solution of hydrogen peroxide was employed, which was found by titration with permanganate to contain 0·0042 grammes

of hydrogen peroxide per c.c. Each c.c. of this solution is, therefore, capable of evolving 2·74 c.c. of oxygen (N.T.P.) when shaken with acid permanganate. In a direct experiment carried out in a nitrometer, 5 c.c. of this peroxide solution yielded 13·9 c.c. of oxygen. Calculated 13·7.

When treated with an equal volume of 20 per cent. aqueous soda and 2 c.c. of 40 per cent. formaldehyde solution (a large excess), 5 c.c. of the peroxide yielded 13·7 and 13·8 c.c. of pure hydrogen (N.T.P.) in two experiments.

The relation between the acid produced and the hydrogen peroxide was ascertained by determining the loss of alkalinity of the soda employed. It was thus found that 1·25 c.c. of normal alkali were required to neutralise the formic acid produced by 5 c.c. of the hydrogen peroxide solution. Calculated 1·2 c.c.

The formic acid produced was also distilled off from the acidified solution, and identified by the usual tests.

The hydrogen evolved was submitted to analysis; 13 c.c. of the gas underwent no contraction when exposed to phosphorus, and on explosion with air gave a contraction corresponding with 12·9 c.c. of hydrogen, no carbon dioxide being produced.

It appears from this that a volume of hydrogen exactly equal to that present in the peroxide is evolved, whilst the corresponding amount of oxygen combines with the aldehyde to produce formic acid.

The reaction between hydrogen peroxide and an excess of formaldehyde in presence of caustic soda, therefore, yields a volume of hydrogen which is exactly equal to the volume of oxygen evolved by the same amount of hydrogen peroxide with acid permanganate. The reaction with formaldehyde can, therefore, be used for the estimation of hydrogen peroxide in the nitrometer. For this purpose the hydrogen peroxide is placed in the nitrometer, an equal volume of 20 per cent. aqueous soda is added, and 2 or 3 c.c. of formalin then run in. The reaction proceeds very rapidly, and the hydrogen is measured in the usual way.

The reaction only proceeds slowly and incompletely unless a large excess of alkali is present. This is shown in the

following experiments, in which 5 c.c. of a dilute solution of hydrogen peroxide were treated with 2 c.c. of formalin solution and 5 c.c. of aqueous soda of varying strengths, which are expressed in their equivalents of a normal solution:—

	Normal Soda.	*Gas Evolved.*	
(1)	5 c.c.	13·8	Evolution very slow.
(2)	10 c.c.	14·8	,, ,,
(3)	15 c.c.	15·3	Evolution slow.
(4)	20 c.c.	15·3	,, ,,
(5)	25 c.c.	15·6	Evolution rapid.
(6)	50 c.c.	15·6	,, ,,

In experiment 6, 10 c.c. of soda solution were employed. The same reaction occurs when sodium carbonate solution is employed instead of caustic soda, but the evolution of the gas is much less rapid.

When a dilute solution of formaldehyde is treated with aqueous soda and an excess of hydrogen peroxide, the reaction takes place slowly and appears not to be complete, even after vigorous and continued shaking for some time. The gas evolved, moreover, contains oxygen derived from the decomposition of the excess of hydrogen peroxide.

After the completion of the work recorded in this paper, my attention was called to the fact that the evolution of hydrogen when formaldehyde is treated with alkaline hydrogen peroxide had already been noticed and described by Blank and Finkenbeiner (Ber., 1898, 31, 2979).

These chemists paid attention exclusively to the amount of formic acid produced, and of caustic soda neutralised when the aldehyde is treated with an excess of alkaline hydrogen peroxide. They found that the amount of formaldehyde present in a solution of moderate concentration can be ascertained by titrating the amount of alkali neutralised in this reaction.

As the foregoing experiments on the action of hydrogen peroxide on formaldehyde confirm the equation established by Blank and Finkenbeiner, and, moreover, deal almost entirely with the volume of hydrogen evolved in presence of an excess of formaldehyde, it has been thought advisable to include a brief account of them.

A reaction of a somewhat similar character is described by Loew (Ber., 20, 145), who found that when formaldehyde is gently warmed with caustic soda and cuprous oxide, hydrogen is vigorously evolved and formic acid produced. Loew regarded the reaction as a catalytic one, and supposed that metallic copper was first produced, which then by virtue of its " specific state of vibration " modified the course of the normal reaction between the aldehyde and hot concentrated caustic soda, viz., the production of methyl alcohol and formic acid.

Quantitative experiments, however, show that the reaction is perfectly definite, two atoms of hydrogen being evolved in the form of free hydrogen for every molecule of cuprous oxide reduced.

$$Cu_2O + 2\,NaOH + 2\,CH_2O = H_2 + 2\,Cu + 2\,H.CO_2Na + H_2O.$$

A quantity of cuprous oxide was warmed with excess of formaldehyde and caustic soda; 25·7 c.c. of hydrogen (N.T.P.) were evolved and 0·1573 grammes of copper produced (weighed as cupric oxide). The amount of hydrogen calculated from the foregoing equation is 27·6 c.c.

Cupric oxide also oxidises formaldehyde in presence of caustic soda with production of free hydrogen, and in this case, as in that of cuprous oxide, each atom of oxygen liberates two atoms of hydrogen.

$$CuO + 2\,NaOH + 2\,CH_2O = Cu + H_2 + 2\,H.CO_2Na + H_2O.$$

Thus 0·2003 grammes of the oxide, prepared by the ignition of the nitrate, yielded 57·1 c.c. of hydrogen (N.T.P.), the calculated amount being 56·36 c.c.

The reaction begins when the mixture is slightly warmed and then proceeds of itself until the reduction is complete.

It is impossible to determine accurately the amount of formic acid produced by the oxidation, because at the temperature necessary for the reduction of the copper oxide the excess of formaldehyde is attacked by the alkali with the production of sodium formate.

The behaviour of a copper salt to formaldehyde and caustic soda is extremely characteristic. When an excess

of soda is mixed with a solution of copper sulphate and formaldehyde then added and the solution gently warmed, cuprous oxide is produced without any evolution of gas. If the temperature be then raised, the cuprous oxide is reduced to copper and a volume of hydrogen is evolved which is equivalent to only half of the oxygen present in the original cupric oxide. If, on the other hand, the copper solution be boiled with caustic soda, so that the black hydroxide, $2\,CuO + Cu(OH)_2$ is formed, and the liquid be then cooled and treated with formaldehyde, no reduction to cuprous oxide occurs and the hydrogen which is evolved is equivalent to the whole of the oxygen of the cupric oxide. Twice as much hydrogen is, therefore, evolved from the black hydroxide as from the blue hydroxide.

The following experiments were carried out with a quantity of copper sulphate which should yield 44·9 c.c. of hydrogen from its copper as cupric oxide, or 22·45 c.c. from its copper as cuprous oxide :—

Cubic Centimetres of Hydrogen (N. T. P.) obtained.

(1) Blue hydroxide	20·4	Mean 22·1
(2) Blue hydroxide	23·9	
(3) Black hydroxide	44·5	Mean 45·5
(4) Black hydroxide	45·8	
(5) Black hydroxide	46·1	
(6) Blue hydroxide		The formaldehyde was added and the heat at once applied, 36·9 c.c. of gas being obtained. In this case the blue hydroxide was partly reduced to cuprous oxide and partly converted into the black hydroxide.

The evolution of hydrogen has also been observed with the peroxides of sodium and barium and with lead dioxide, and the quantitative relations of these reactions are at present being investigated.

On the other hand, the oxides of bismuth and mercury are reduced by alkaline formaldehyde without any evolution of gas. Manganese dioxide appears to have no effect.

Great interest, from the theoretical point of view, attaches to the question whether the hydrogen which is evolved arises from the aldehyde itself or from the oxidising agents employed. If the reaction be regarded as essentially due to hydrogen peroxide, two views are possible. In the

first place, it may be supposed that the reaction consists in the direct substitution of hydrogen by hydroxyl.

$$2\,O{:}C\begin{matrix}H\\H\end{matrix} + \begin{matrix}HO\\HO\end{matrix} = 2\,O{:}C\begin{matrix}H\\OH\end{matrix} + H_2.$$

On the other hand, the reaction may be compared with the reducing action exerted by hydrogen peroxide on such substances as permanganic acid, silver oxide, etc., which has been interpreted by many as due to the decomposition of the peroxide into oxygen, which is evolved, and hydrogen which at once reduces the oxidising agent which is present. The reaction of hydrogen peroxide on formaldehyde would be the inverse of this, the hydrogen being in this case evolved, whilst the oxygen goes to oxidise the reducing substance present.

The fact that a similar reaction is given by the two oxides of copper would then be explained on the supposition that in these cases a molecule of water also enters into the reaction, the free hydrogen being derived from this molecule.

On the other hand, it must be remembered that the hydrogen atoms in a compound like formaldehyde or formic acid are in a peculiarly mobile condition, as is shown by such reactions as the oxidation of formic acid by silver oxide, and by the formation of sodium oxalate and free hydrogen when sodium formate is heated. The production of free hydrogen and formic acid from formaldehyde may be supposed to occur in two stages, two atoms of hydrogen being replaced in two molecules of the aldehyde by one atom of oxygen,

$$(1)\quad 2\,OC\begin{matrix}H\\H\end{matrix} + O = OCH{\cdot}O{\cdot}HCO + H_2.$$

and the unstable intermediate product thus produced being at once hydrolysed by the caustic alkali present,

$$(2)\quad OCH{\cdot}O{\cdot}HCO + H_2O = 2\,OCH{\cdot}OH.$$

This view has the advantage of explaining the action of hydrogen peroxide and the metallic oxides on precisely the same grounds. Further experiments on the subject are in progress, with the object of obtaining more direct evidence on this point.

The foregoing paper was read before the Chemical Society on June 15, 1899, and an abstract of it appeared in the Proceedings of the Society, 1899 (xv., 158).

the pus remained unaffected. Aerobic and anaerobic cultivations remained sterile.

The prediction was made that the case was probably one of tropical abscess, and that the *amœba coli* would be found in the discharge in a day or two.

This prediction was verified three days later, when *amœbæ coli* were found to be numerous in the discharge.

The amœba coli is mainly found about the walls of the abscess cavity, and is frequently absent from the bulk of the contents of the abscess. After draining, when the walls of the abscess cavity begin to break down, the amœba coli makes its appearance in the discharge.

IV.

The Early Diagnosis of Diphtheria.

A microscopical examination of cultures of swabbings, &c., on Loeffler's blood serum for diphtheria diagnosis will frequently show the presence of the diphtheria bacillus in as short a time as six or seven hours. Cultures made between 9 and 10 a.m., and incubated at 37° C., may thus be examined from 4 to 6 p.m., and valuable time saved. It is rarely possible to find any visible colonies in so short a time; a scraping is therefore taken from the whole surface of the medium, and a cover-glass preparation made and stained with Loeffler's blue, keeping the material to a small area at the centre of the cover-glass, and not spreading. If no bacilli be detected a report cannot be given, but the tube must be replaced in the incubator, and examined next morning. In this way 252 specimens have been recently examined. Of these, the bacillus was found in thirty-six, and not found in 216, or 14·3 per cent. positive. Of the 216 in which it was not found, 104 were found to be positive on examination next day. It follows, therefore, that about 25·5 per cent. of cases yielding a positive result on bacteriological examination for diphtheria can be detected and reported on within six or seven hours of receiving the swabbing or membrane.

Another point of interest arising from these early diagnoses is the fact that streptococci are found to be

present, more or less abundantly, frequently the latter, and forming beautiful chains, in the majority of cases examined.

This raises the question as to whether any significance can be attached to the presence of streptococci in the culture tubes in diphtheria diagnosis.

Another point which is suggested by this fact is that for "streptococcus diagnosis" (blood, &c.) the best results may be obtained by inoculating Loeffler's serum, incubating at 37° C. for six to seven hours, and then examining. In this way contaminating organisms may be to a large extent excluded.

V.

Table showing the Age-Incidence of Diphtheria.

Based on the examination of 1,116 specimens received at the Jenner Institute for diagnosis.

Years.	No. of Cases, Positive.	No. of Cases, Negative.	Percentage of Positive Cases.
1	24	24	50
2	21	19	52·5
3	44	29	60·27
4	47	32	59·5
5	58	30	66
6	37	34	52·1
7	42	26	61·77
8	28	24	53·85
9	22	32	40·74
10	23	32	41·8
11	23	24	48·93
12 to 13 ...	38	42	47·5
14 ,, 15 ...	17	35	32·7
16 ,, 20 ...	27	54	33·3
21 ,, 25 ...	32	53	37·65
26 ,, 30 ...	15	38	28·3
31 ,, 35 ...	6	25	19·35
36 ,, 40 ...	9	18	33·3
41 ,, 50 ...	8	13	38·1
51 ,, 60 ...	2	5	28·57
61 ,, 80 ...	1	3	25

R. T. HEWLETT.

VI.

Flagella Staining.

It is somewhat surprising that the night-blue method of staining flagella, described by McCrorie, of Glasgow, has not been more universally adopted. The process is short and easy, while the results are better and more certain than those obtained by any other process yet described.

I have adopted the following method, which is a modification of the original:—Slides are used in preference to cover-slips, as they are more easily cleaned and manipulated. These are washed as clean as possible, then dipped in 25 per cent. nitric acid, and afterwards kept in methylated spirit. The slides should be quite free from greasiness, so that water will spread on the surface without separating into drops. A twenty-four hours surface agar culture is used. A small portion of the growth is removed, and suspended in a few drops of sterilized tap water in a watch glass. For some reason or other tap water gives better results than distilled water. The suspension must be made by stirring very gently with the needle, or better, by agitating the watch glass till the germs spread out through the water. A drop or two of the suspension is placed on a slide, and made to spread over a portion of the surface by turning the slide about—not by using the needle. The excess is soaked up by blotting paper, and the films allowed to dry at the room temperature. The film is *not fixed*, as this appears to injure the flagella, and it is not necessary, as the germs will be found to adhere to the slide.

The stain is made by dissolving 1 grm. of tannic acid, 1 grm. of potash alum in 40 c.c. distilled water, and adding to this ·5 grm. of night-blue dissolved in 20 c.c. absolute alcohol. A copious precipitate falls down, which is filtered out. The filtrate is the stain.

The slides prepared as above are stained with this for two minutes, and then washed gently in running water. If the preparation be now dried on blotting-paper and examined with the microscope, it will be found that the flagella are

stained, but that the germ itself is not stained at all, or only very slightly. If the staining process be allowed to go on longer, the germ will stain, but a copious precipitate forming on the slide will obscure the preparation. To obviate this, a contrast stain is used for the germ. The best reagent was found to be aniline water gentian violet, which gives a very good contrast to the blue flagella. If the slides be stained in this for one or two minutes, the germs will take up the gentian violet. If the preparations are to be kept, a cover-slip can be fixed with Canada balsam over the film on the slide.

The staining by night-blue is evidently caused by precipitation, and the drawback to the method is, that the precipitate sticks firmly to the glass slide, and does not wash off; so that the spaces between the germs are not clear. This does not make much difference, as parts of the preparation will usually be found clear enough for all purposes. But, to get rid of this precipitate as much as possible, the stain should always be filtered on to the prepared slide. During the two minutes of staining the stain should be changed two or three times. Or the preparation may be stained deeply—say for ten minutes—and then decolourised to the necessary extent by dipping very rapidly into dilute alcohol—equal parts of methylated spirit and water.

By this method I have succeeded in staining flagella on bacillus coli, typhosus, prodigiosus, proteus vulgaris, subtilis, tetani and choleræ, and spirillum rubrum. Good preparations have been made from a coli culture after ten days. Proteus vulgaris seems to give the finest preparations.

I consider that the application of heat, and the spreading of the film with the needle are very fatal to good results in flagella staining.

On examining a preparation of proteus vulgaris, stained by this method, with a very high magnifying power, some blue spots were seen in the purple-stained bodies of the germs. It is suggested that these may be the proximal ends of flagella, which are "end on" to the observer.

N. MORTON.

VII.

Capsule Staining.

The usual methods recommended for demonstrating the capsules surrounding microbes depend for their success on the difference in affinity for the colouring reagent shown by the microbe and its investing capsule; they are, in fact, single-colour methods, in which the microbe appears deeply-stained, lying within a capsule of much lighter tint. Obviously, such preparations would be greatly improved if markedly contrasting colours could be substituted, and to a certain extent, at least, this can be effected by means of the "night blue" staining reagent (described by Dr. Morton in the note on flagella staining), counter-staining with ordinary carbol-fuchsin. At present this method has been successful with cultures on LIQUID serum of the diplococcus pneumoniæ, Friedländer's pneumo-bacillus and M. tetragenus. The *modus operandi* adopted is as follows:—A little of the deposit in the serum culture tube is spread on a clean slide, allowed to dry, fixed with dilute acetic acid and washed in distilled water. The preparation is then stained with carbol-fuchsin for about a minute, washed again in distilled water and dried. Finally, the "night blue" stain is applied, allowed to act for one to two minutes, aided, perhaps, by gentle heating, then washed and dried.

Owing to the great tendency of the "night blue" stain to form a dense precipitate, one at times fails to obtain good preparations, but when successful, the result is very striking, the microbe appearing stained dark-red, surrounded by a well-defined, blue-coloured capsule. Liquid serum forms the best medium for demonstrating capsule formation by pathogenic bacteria.

A. MOORE.

VIII.

Disinfection on a large scale with Formalin.

A factory using a large amount of gelatin for a commercial purpose, in which it was absolutely necessary that the gelatinised product should be clear and free from

opacities, suddenly found itself in great difficulties owing to the appearance of such opacities in the finished product in large numbers. The difficulty had been continuous for some months and had assumed very serious proportions. The known and available remedies were exhausted without effect, and various remedies, suggested by chemical experts, were tested with negative results. The Institute was applied to for assistance. A careful examination of the opacities lent support to the suggestion that they were of mycological origin, as several of the spots contained fragments of the mycelium of a mould. A local examination further showed that the walls of the building harboured a luxuriant growth of a sporing mould. The artificial inoculation of the spores of this mould into the unfinished gelatinous product produced very similar appearances to those met with in the finished product. It was recommended that the walls of the building be freed from all traces of the mould. This was done by the use of formalin in the form of a hot spray, and the source of the trouble disappeared. It is doubtful whether any other method of disinfection would have been so successful with such resistent forms of life. Formalin, as is well known, possesses high disinfectant properties, and we have probably no more efficacious method at our command for the disinfection of large surfaces than this agent when employed in the form of a fine hot spray liberated under pressure. This latter condition introduces the additional effects due to heat and mechanical cleansing.

A. M.
S. R.

[illegible]

[illegible] water. The box is provided with inlet and outlet [illegible] and *d*. A regulating valve [illegible] is attached to *a*, and [illegible] are openings provided with screw caps. The water [illegible] flask *B* is heated up to 60° to 70° C., and after [illegible] through the hot stage escapes at *G*.

The object to be examined is placed on a long glass [illegible] the ends of which project through apertures in the [illegible] box and are held in position by two springs. In the [illegible] of the metal box is a tubular aperture to allow [illegible] objective coming close to the object.

The means of regulating the temperature are ind[illegible] in fig. 2.

A is the outer case of the metal box. The water [illegible] ing the cavity of the box through tube *a* passes th[illegible]

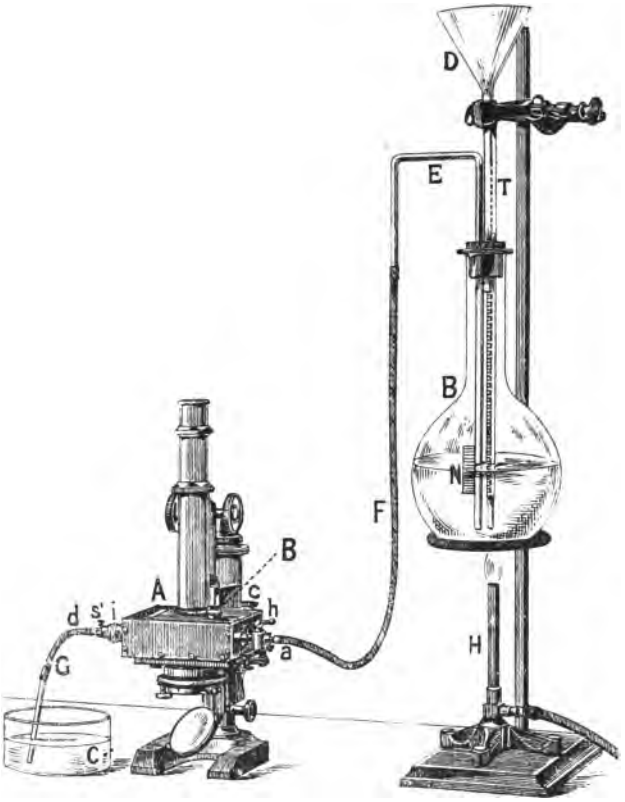


FIG. 1.

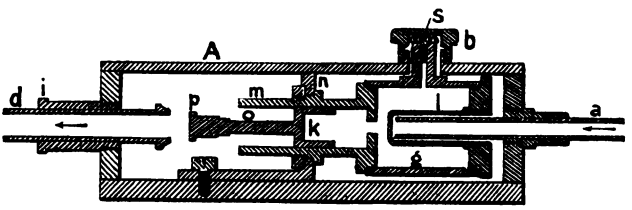


FIG. 2.

the annular space of the closed cylinder *l*, which projects into an airchamber *g*. The air in this chamber in consequence assumes a definite volume for every temperature of water entering through *a*. The pressure of the air in *g* and *l* is kept constant by the movable piston *k*, which slides freely in the tube *m*, which is in free communication with *g* and *l*. Consequently every temperature of water entering through *a* corresponds to a definite position of the piston *k*. The piston carries at the end of its rod *o* a flat plate *p*, which is capable of closing the orifice of the exit tube *d*. The exit tube *d* is capable of sliding in the direction of its axis through the watertight gland *i*. It is also capable of a more delicate adjustment of its position by means of a graduated screw collar working on *i*, which serves the purpose of a fine adjustment. To set the instrument for any given temperature, it is only necessary to allow water of a higher temperature than that required to flow through the apparatus from *a* to *d*, and when the water has attained the requisite temperature to slide in *d* until the exit of water is arrested. As the temperature falls by radiation, the volume of air in *g* and *l* will diminish, the piston *k* will travel in the direction of *p*, *o*, *k*, *l*, and *a*, thus opening the exit tube by the withdrawal of plate *p*. This permits a fresh supply of hot water to flow through the instrument, which, expanding the air in *g* and *l*, forces out the piston *k*, and again diminishes the flow of water and so on.

In this manner one can quickly obtain any temperature desired between 20° and 60° C. The stage contains a thermometer which indicates the temperature of the box. The temperature of the object under examination is about two degrees lower than that indicated by the thermometer. The variations in temperature of the instrument are not greater than $\frac{1}{5}$ to $\frac{1}{10}$ of a degree.

A. MACFADYEN.

II.

A New Photomicrographic Apparatus.

The illustration on the accompanying page represents the photomicrographic apparatus which has been designed

A NEW PHOTOMICROGRAPHIC APPARATUS.

for the Institute by the writer. The objects aimed at in the design were to combine perfect rigidity with facility of manipulation and the certain possibility of being able to reproduce at any time experimental conditions previously determined.

The points of novelty in this instrument may be best described under the following headings :—

(1) *Rigidity.*—The entire instrument is supported on a cast iron girder base, which combines the maximum of strength with the minimum of weight.

(2) *Focussing.*—This is accomplished in a novel manner. Running the entire length of the base of the instrument is a steel rod, provided with movable brass heads at intervals. At the end of this rod, immediately under the pivot which supports the swinging tail-piece which carries the microscope and illuminating apparatus, is a grooved pulley carrying a silk cord on which a uniform tension is maintained by means of weights. This cord passes up the supporting pivot of the tail piece, which is made hollow for this purpose, and is led over guide pulleys to the fine adjustment of the microscope. By means of this arrangement it is possible to swing aside the entire microscope and illuminating apparatus for the purpose of examining the preparation with the naked eye without in any way interfering with the fine adjustment arrangements.

(3) *Camera extension.*—The leather bellows has been made in segments, so that either the entire length of bellows extension may be employed or any fraction thereof. The mahogany frames which support the bellows at intervals rest, at their edges only, on two brass tubes running the entire length of the girder base, one on each side. The contact of the frames with these guiding tubes is alternately V and flat. The clamping arrangements are entirely independent, and thus admit of no possibility of disturbance of the accuracy of alignment.

(4) *The microscope stand.*—The form of microscope stand chosen is known as the Wales' model, which permits of the instrument being placed at any inclination while still preserving its centre of gravity approximately uniform in

position. The fine adjustment is of Swift's side lever variety, a mechanical adjustment of great delicacy, and one on which any pressure on the screw makes the least possible alteration in the alignment of the microscope tube. The stage is a large plain revolving one provided with centering screws, but without other mechanical adjustments. The substage condenser is an apochromatic combination by Swift, provided with rack and pinion focussing adjustment, and with centering screws; it is movable at will, being attached in a dovetail fitting.

(5) *The illuminating arrangements.*—Mounted on the swinging tail-piece of the apparatus, and separated from the microscope by a light screen, provided with an Iris diaphragm, is a horizontal brass tube which serves the purposes of an optical bench. On this are mounted an arc lamp and a parallelising system. The arc lamp is of a special form devised by the writer and Mr. Carver, a full description of which will be found in the *Journal of the Royal Microscopical Society*, page 170, 1898. It will suffice here to say that the lamp is of the hand-fed variety, and that centering is maintained by regulating the position of the arc so as to project its image by means of a pin-hole camera on to the intersection of two cross wires with which the instrument is provided.

(6) The entire instrument rests on three levelling screws supported by thick lead discs on the surface of a stone table which is carried by brick supports passing through the floor to the concrete foundation of the building.

This instrument has proved convenient in working, and easy to manipulate. It is unencumbered with mechanical accessories and when once adjusted no further alterations are required.

J. E. BARNARD.

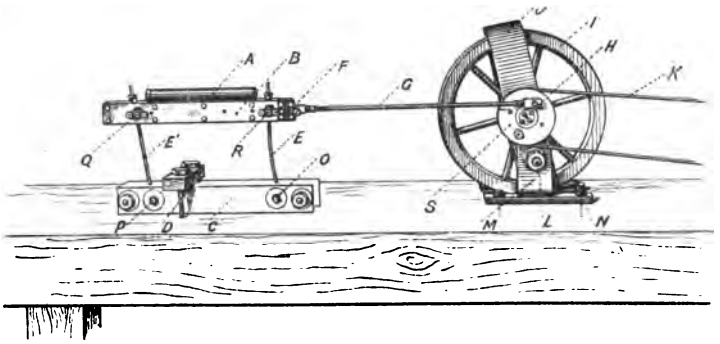
III.

A Method of Rapidly Disintegrating Micro-organisms.

In the course of a series of investigations now in progress on the separation of the intracellular contents of pathogenic bacteria, it became necessary to disintegrate the organisms thoroughly and in as short a time as possible.

It was found that by shaking a mass of the organisms with quartz sand and hard steel balls, complete disintegration of any mass could be obtained, if the shaking was violent enough, in as short a time as ten minutes.

It accordingly became necessary to devise a form of shaking machine that would accomplish the object. The arrangement shown in the illustration has been found so satisfactory that a brief description may be of interest. A, is a steel tube closed at each end into which the mixture is introduced. It is rigidly attached to an aluminium frame B, which, by means of the rod G, and the crank pin H, is caused to oscillate horizontally. Two steel rods E, E′, support the frame B, and, by means of the joints Q, R, P, and O, allow of parallel motion. C, is a frame of steel clamped to the table by D, and carrying the joints P and O. Motion is



given to the crank wheel S, by means of a cord K, driven by an electro-motor. The axle of S runs in bearings supported in the frame J, and carries, friction tight only, a fly-wheel I. The frame J swings about the stud L, carried in a pair of angle pieces M, and bolted to the table by N. This arrangement serves to allow of the cord K being easily adjusted for tension.

Running the arrangement at 3,500 revolutions per minute, complete disintegration of the tubercle bacillus is accomplished in ten minutes. In practice, however, it is preferable to run at a slower rate and thus save wear and tear at a slightly increased expenditure of time.

SYDNEY ROWLAND.

IV.

A New Microscope Lamp.

The employment of electricity for the purposes of microscopical illumination has always proved a difficult task. An ordinary incandescent lamp is quite unsuitable, owing to the projected image of the filament appearing in the field of view. It seemed, however, likely that if a suitable white surface could be prepared and strongly illuminated by an incandescent lamp, it could be used as a source of illumination of sufficient intensity for microscope work. In the illustration which accompanies this note this has been carried out in the following manner :—

The lamp is enclosed in a brass tube cut off obliquely at one end, and provided with an aperture opposite the oblique surface. The entire inner surface of the tube is coated with a thick layer of oxide of zinc, which when the lamp is incandescing, gives off a perfectly white light of sufficient intensity for most purposes of routine microscopical investigation. In this way the objectionable image of the filament is entirely got rid of, and the field of the microscope becomes illuminated with a soft white light very pleasant to the eye.

To those who are accustomed to work with the classical critical illumination of the sharply projected image of the flame, the new light will at first appear confusing, but if the substage condenser be so adjusted as to give the maximum illumination it will be obvious that at this point the image is a critical one, although the surface of the zinc oxide is too perfect to present any definite appearance in the field of view. The intensity of the light is ample, even for working with a one-twelfth oil immersion objective and a low power eye-piece.

J. E. BARNARD.

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